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Inventor(s)/Applicant(s)

Last Name	First Name	Initial	Residence Address (City & State or Foreign Country)
Simonet	W. Scott		2293 Watertown Court, Thousand Oaks, California
Asuncion	Franklin		2279 McDonald Street, Simi Valley, California
Min	Hosung		5811 Woodman Avenue, #1, Sherman Oaks, California
Jing	Shuqian		3254 Bordero Lane, Thousand Oaks, California

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GRNF4 A NEUROTROPHIC FACTOR

Enclosed application parts are:

- ☒ 78 pages of specification, 0 pages of sequence listing, 10 pages of claims and 1 page of abstract, totaling 89 pages
- ☒ 20 sheets of drawings
- ☒ Other Declaration and Power of Attorney
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Respectfully submitted,

Daniel R. Curry

Daniel R. Curry
Attorney for Applicants
Registration No.: 32,727
Phone: (805) 447-8102
Date: December 9, 1998

Please send all future correspondence to:
U.S. Patent Operations/DRC
Dept. 430, M/S 27-4-A
AMGEN INC.
One Amgen Center Drive
Thousand Oaks, California 91320-1799

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GRNF4

A NEUROTROPHIC FACTOR

1. Field of the Invention

The present invention relates to neurotrophic factors. In particular, the invention relates to novel proteins or polypeptides related to glial cell line-derived neurotrophic factor (GDNF). The molecules are designated GRNF4 for GDNF-related neurotrophic factor 4. The invention further relates to molecules comprising polynucleotides encoding and amino acid sequences constructing the neurotrophic factors as well as pharmaceutical compositions containing such molecules.

2. Background of the Invention

Glial Cell line-Derived Neurotrophic Factor

Glial cell line-derived neurotrophic factor (GDNF) was initially isolated and cloned from rat B49 cells as a potent neurotrophic factor that enhances survival of midbrain dopaminergic neurons (Lin et al., Science, 260, 1130-1132, 1993). Studies have indicated that this molecule exhibits a variety of other biological activities, having effects on several types of neurons from both the central and peripheral nervous systems. In the central nervous system (CNS), GDNF has been shown to prevent the axotomy-induced death of mammalian facial and spinal cord motor neurons (Li et al., Proceedings Of The National Academy Of Sciences, U.S.A., 92, 9771-9775, 1995; Oppenheim et al., Nature, 373, 344-346, 1995; Yan et al., Nature, 373, 341-344, 1995; Henderson et al., Science, 266, 1062-1064, 1994; Zurn et al., Neuroreport, 6, 113-118, 1994), and to rescue developing avian motor neurons from natural programmed cell death (Oppenheim et al., 1995 supra). Local administration of GDNF has been shown to protect nigral dopaminergic neurons from axotomy-induced (Kearns and Gash, Brain Research, 672, 104-111, 1995; Beck et al., Nature, 373, 339-341, 1995) or neurotoxin-induced degeneration (Sauer et al., Proceedings Of The National Academy Of Sciences U.S.A., 92, 8935-8939, 1995; Tomac et al., Nature,

373, 335-339, 1995). In addition, local administration of GDNF has been shown to induce sprouting from dopaminergic neurons, increase levels of dopamine, noradrenaline, and serotonin, and improve motor behavior (Tomac et al., 1995 supra).

More recently, GDNF has been reported to be a potential trophic factor for brain noradrenergic neurons and Purkinje cells. Grafting of fibroblasts ectopically expressing GDNF prevented 6-hydroxydopamine-induced degeneration and promoted the phenotype of adult noradrenergic neurons in vivo (Arenas et al., Neuron, 15, 1465-1473, 1995), while exogeneously applied GDNF effectively promoted survival and morphological differentiation of embryonic Purkinje cells in vitro (Mount et al., Proceedings Of The National Academy Of Sciences U.S.A., 92, 9092-9096, 1995). In the peripheral nervous system, GDNF has been shown to promote the survival of neurons in nodose, ciliary, and sympathetic ganglia, as well as small populations of embryonic sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia (Trupp et al., Journal Of Cell Biology, 130, 137-148, 1995; Ebendal et al., Journal Of Neuroscience Research, 40, 276-284, 1995; Oppenheim et al., 1995 supra; Yan et al., 1995 supra; Henderson et al., 1994 supra). GDNF has also been reported to enhance the expression of vasoactive intestinal peptide and preprotachykinin-A mRNA in cultured superior cervical ganglion (SCG) neurons, and thus, GDNF effects the phenotype of SCG neurons and induces bundle-like sprouting (Trupp et al., 1995 supra).

Expression of GDNF has been observed in a number of different cell types and structures of the nervous system. In the CNS, GDNF mRNA expression has been observed by reverse transcriptase polymerase chain reaction (RT-PCR) in both developing and adult rat striatum, the major target of nigral dopaminergic innervation. GDNF mRNA expression has also been observed in other regions, including hippocampus, cortex, thalamus, septum, cerebellum, spinal cord, and medulla oblongata (Arenas et al., supra 1995; Poulsen et al., Neuron, 13, 1245-1252, 1994; Springer et al., Experimental Neurology, 127, 167-170, 1994; Stroemberg et al., Experimental Neurology, 124, 401-412, 1993; Schaar et al., Experimental Neurology, 124, 368-371, 1993). In human, GDNF transcripts have also been detected in the striatum, with the highest level in the caudate and lower levels in the putamen. Detectable levels are also found in the hippocampus, cortex, and spinal cord, but not in the cerebellum (Schaar et al., Experimental Neurology, 130,

387-393, 1994; Springer et al., 1994 supra). In the periphery, GDNF mRNA expression has been reported in DRG and SCG of postnatal day 1 rats, sciatic nerve, and primary cultures of neonatal Schwann cells (Trupp et al., 1995 supra; Hoffer et al., Neuroscience Letters, 182, 107-111, 1994; Henderson et al., 1994 supra; Springer et al., 1994 supra). In addition, recent studies have shown that GDNF transcripts are widely expressed in peripheral non-neuronal organs, including postnatal testis and kidney, embryonic whisker pad, stomach, and skin. Expression can be detected at lower levels in embryonic muscle, adrenal gland and limb bud, and in postnatal lung, liver and ovary (Trupp et al., 1995 supra; Henderson et al., 1994 supra).

Detailed descriptions of the preparation and characterization of GDNF polypeptides may be found in U.S. Patent Application No. 08/182,183 filed May 23, 1994 and its parent applications (also see PCT/US92/07888, WO 93/06116 filed September 17, 1992 and European Patent Application No. 92921022.7, Publication No. EP 610 254) the disclosures of which are hereby incorporated by reference. Additional GDNF polypeptides are described in WO 9711964 (U.S. Patent Application No. 08/535,681 filed September 28, 1995; PCT/US96/14915), the disclosure of which is hereby incorporated by reference. Other neurotrophic factors that are structurally related to GDNF include a protein referred to as "neurturin" (described in Nature, 384(5):467-470, 1996; and WO 9708196) and a protein referred to as "persephin" (Milbrandt et al., Neuron 20(2):245-253, 1998; and WO 9733911), the disclosures of which are hereby incorporated by reference.

GDNF Therapy

GDNF therapy is helpful in the treatment of nerve damage caused by conditions that compromise the survival and/or proper function of one or more types of nerve cells. Such nerve damage may occur from a wide variety of different causes. Nerve damage may occur to one or more types of nerve cells by: (1) physical injury, which causes the degeneration of the axonal processes and/or nerve cell bodies near the site of injury; (2) temporary or permanent cessation of blood flow to parts of the nervous system, as in stroke; (3) intentional or accidental exposure to neurotoxins, for example, chemotherapeutic agents (e.g., cisplatinum) for the treatment of cancer or dideoxycytidine (ddC) for the treatment of AIDS; (4) chronic metabolic diseases, including diabetes or

renal dysfunction; or (5) neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS), which result from the degeneration of specific neuronal populations.

Several studies indicate that GDNF therapy is particularly helpful in the treatment of neurodegenerative conditions such as the degeneration of the dopaminergic neurons of the substantia nigra in Parkinson's disease. The only current treatments for Parkinson's disease are palliative, aiming at increasing dopamine levels in the striatum. The expected impact of GDNF therapy is not simply to produce an increase in the dopaminergic neurotransmission at the dopaminergic nerve terminals in the striatum (which will result in a relief of the symptoms), but also to slow down, or even stop, the progression of the degenerative processes and to repair the damaged nigrostriatal pathway and restore its function. GDNF may also be used in treating other forms of damage to or improper function of dopaminergic nerve cells in human patients. Such damage or malfunction may occur in schizophrenia and other forms of psychosis. Current treatments for such conditions are symptomatic and require drugs which act upon dopamine receptors or dopamine uptake sites, consistent with the view that the improper functioning of the dopaminergic neurons which innervate these receptor-bearing neuronal populations may be involved in the disease process. In spite of the continued discovery of neurotrophic factors and the continuing research involving therapeutic compositions in this field, compounds for the treatment of nerve damage and/or the enhancement of proper nerve function are still needed.

SUMMARY OF THE INVENTION

The neurotrophic factor polypeptides of the present invention are designated herein as GDNF-related neurotrophic factor 4 (GRNF4) protein products, denoting the status as a fourth member of what has been referred to as the GDNF family of structurally related neurotrophic factors. The novel molecules are functionally characterized by the ability to bind GDNF family receptor-alpha-3 (GFRα-3) (i.e., GRNF4 activity). The novel proteins and polypeptides also provide part of a molecular complex which mediates or induces

phosphorylation of tyrosine residues of the Ret receptor protein tyrosine kinase. Exemplary GRNF4 protein products comprise an amino acid sequence selected from the group consisting of: an amino acid sequence of Figure 3 (SEQ ID NO:__), an amino acid sequence of Figure 7 (SEQ ID NO:__) and consensus sequences such as those depicted in Figure 18 (SEQ ID NO:__).

In one aspect, the present invention provides for the production of GRNF4 protein products by means of recombinant or genetic engineering techniques. In alternative embodiments, the GRNF4 protein products are synthesized by chemical techniques, or produced by a combination of genetic engineering and chemical techniques.

In another aspect of the present invention, the GRNF4 protein products may be made in glycosylated or non-glycosylated forms. Derivatives of GRNF4 proteins and polypeptides typically involve attaching a GRNF4 molecule to a water soluble polymer. For example, a GRNF4 protein or polypeptide may be conjugated to one or more polyethylene glycol molecules to decrease the precipitation of the GRNF4 protein product in an aqueous environment.

Yet another aspect of the present invention includes the various polynucleotides encoding GRNF4 protein products. These polynucleotides are used in the expression of GRNF4 in eukaryotic or prokaryotic host cells, wherein the expression product or a derivative thereof is characterized by the ability to bind GFRα-3, and to act as part of a molecular complex which mediates or induces phosphorylation of tyrosine residues of the Ret receptor protein tyrosine kinase. The polynucleotides and/or the genetically engineered cells may also be used in cell therapy or gene therapy applications. Suitable nucleotide molecules include those specifically depicted in the Figures as well as degenerate sequences, naturally occurring allelic variations and modified sequences based on the present invention. Exemplary polynucleotide molecules include: (a) sequences set forth in Figure 2 (SEQ ID NO. __) or Figure 6 (SEQ ID NO. __); (b) a molecule which (1) hybridizes to a complementary sequence of (a) and (2) encodes an amino acid sequence with GRNF4 activity; and (c) a molecule which but for the degeneracy of the genetic code would hybridize to a complementary sequence of (a) and (2) encodes an amino acid sequence with GRNF4 activity. Also disclosed herein are vectors containing such polynucleotides, wherein the sequences typically are operatively linked to one or more

operational elements capable of effecting the amplification and/or expression of the sequence. Both prokaryotic and eukaryotic host cells containing such vectors are contemplated. Typically, the host cell is selected from mammalian cells and bacterial cells, such as a COS-7 cell or E. coli, respectively. The present invention further includes the recombinant production of GRNF4 protein products wherein transformed or transfected host cells are grown in a suitable nutrient medium, and the GRNF4 protein product expressed by the cells is, optionally, isolated from the host cells and/or the nutrient medium. If bacterial expression is involved, the method may further include the step of refolding the neurotrophic factor. "Transformed or transfected" as used herein refers to cells that are no longer in their naturally occurring form, i.e., the cells have been recombinantly or genetically engineered or modified to express the GRNF4 protein or polypeptide.

The host cell may also be selected for its suitability to human implantation, wherein the implanted cell expresses and secretes a neurotrophic factor of the present invention. The host cell also may be enclosed in a membrane suitable for human implantation. The host cell may be transformed or transfected ex vivo. An exemplary device for treating nerve damage involves: (a) a membrane suitable for implantation; and (b) cells encapsulated within the membrane, wherein the cells express and secrete a neurotrophic factor receptor as disclosed herein. The membrane may be selected from a semipermeable material, i.e., a material that is permeable to the neurotrophic factor but impermeable to materials detrimental to the encapsulated cells.

Exemplary protein products of the present invention include isolated and purified protein products comprising an amino acid sequence as described herein, wherein the protein products bind GDNF family receptor-alpha-3 (GFRα-3). Consensus amino acid sequences may also be derived from these exemplary molecules to provide additional GRNF4 protein products.

Also disclosed herein are pharmaceutical compositions comprising a GRNF4 protein product of the present invention in combination with a pharmaceutically acceptable carrier. A variety of other formulation materials may be used to facilitate manufacture, storage, handling, delivery and/or efficacy.

Another aspect of the present invention includes the therapeutic use of GRNF4 genes and protein products. For example, a circulating or soluble GRNF4 protein product may be used alone or in conjunction with additional agents (for example, including other neurotrophic factors such as GDNF, persephin and/or neurturin) in treating disease of or injury to the nervous system. Thus, the protein products and pharmaceutical compositions of the present invention may be used in treating improperly functioning dopaminergic nerve cells, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. In another embodiment, a recombinant GRNF4 gene may be inserted in the cells of tissues which may benefit from increased sensitivity to GRNF4, such as motor neurons in patients suffering from amyotrophic lateral sclerosis. GRNF4 might also be used in the treatment of peripheral sensory neuropathy or neurological disorders associated with improperly functioning peripheral sympathetic nerves. In yet another embodiment, it is envisioned that GRNF4 may be used to treat diseases associated with bone loss such as osteoporosis, osteogenesis imperfecta or hypercalcemia of malignancy. GRNF4 may affect the development of osteoclasts, osteoblasts or chondrocytes as it was isolated from a cDNA library prepared from osteoporotic bones where these cell types are abundant and actively function to remodel the skeleton.

In a further aspect of the invention, an oligonucleotide probe based on the GRNF4 nucleotide sequence may be used to identify GRNF4-related molecules. In addition, the present invention provides for experimental model systems for studying the physiological role of GRNF4. Such systems include assays involving anti-GRNF4 antibodies or oligonucleotide probes as well as animal models, such as transgenic animals which express high levels of GRNF4 or animals derived using embryonic stem cell technology in which the endogenous GRNF4 genes were deleted from the genome. An anti-GRNF4 antibody will bind a peptide portion of the GRNF4 protein or polypeptide. Antibodies include monoclonal and polyclonal antibodies which may be used for detection and purification of GRNF4 protein products.

Additional aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following description, which details the practice of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a depicts a nucleotide encoding an open reading frame of a clone (smcb2-00011-d2) which showed homology to the C-terminal active domain of GDNF.

Figure 1b depicts a comparison of the smcb2-00011-d2 open reading frame to neurturin.

Figure 1c depicts the full sequence of murine GRNF4 which was obtained by further sequencing the smcb2-00011-d2 clone. The sequence includes GDNF-like homology and 3'-UTR.

Figure 2 depicts a polynucleotide molecule comprising a nucleotide sequence (SEQ ID NO:__) encoding murine GRNF4. The amino acid sequence of a full-length GRNF4 protein product is encoded by nucleotides 217 to 891.

Figure 3 depicts the 224 amino acid sequence (SEQ ID NO:__) of the full length murine GRNF4 protein product.

Figure 4 depicts a comparison of murine GRNF4 and neurturin amino acid sequences. Murine GRNF4 is approximately 39% identical to neurturin

Figure 5 depicts a comparison of the murine GRNF4 amino acid sequence to those of neurturin, persephin and GDNF.

Figure 6 depicts the nucleotide sequence for human GRNF4.

Figure 7 depicts the amino acid sequence for human GRNF4.

Figure 8 depicts an amino acid sequence comparison between mouse and human GRNF4.

Figure 9 depicts a Northern blot analysis of human tissues for human GRNF4.

Figure 10 depicts a Northern blot analysis of mouse tissues for mouse GRNF4.

Figure 11 presents a radioautograph of [^{125}I]-labeled GRNF4 fractionated by a 16% SDS-PAGE under non-reducing (NR) and reducing conditions.

Figure 12 depicts the binding of [^{125}I]-labeled GRNF4 to the surface of NSR-5 cells (genetically engineered mouse neuroblastoma, Neuro-2a, cells that express GFRα-3).

Figure 13 depicts binding of GRNF4 to BiaCore surface coated by a soluble flag-tagged GFRα-3 protein.

Figure 14 depicts chemical cross-linking of [^{125}I]-labeled GRNF4 to the soluble GFRα-3-human Fc fusion protein.

Figure 15 depicts chemical cross-linking of [^{125}I]-labeled GRNF4 to GFRα-3 and Ret receptors expressed in NSR-5 cells.

Figure 16 depicts GRNF4 induced tyrosine phosphorylation of the Ret receptors expressed in NSR-5 cells.

Figure 17 depicts the dose-dependence of GRNF4 induced tyrosine phosphorylation of the Ret receptors expressed in NSR-5 cells (Figure 17, panel A) and the kinetics of GRNF4 induced tyrosine phosphorylation of the Ret receptors expressed in NSR-5 cells (Figure 17, panel B).

Figure 18 depicts a GRNF4 consensus sequence.

DETAILED DESCRIPTION OF THE INVENTION

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor which exhibits a broad spectrum of biological activities on a variety of cell types from both the central and peripheral nervous systems. It is a glycosylated, disulfide-linked dimer which is distantly related (less than 20% homology) to the transforming growth factor-ß (TGF-ß) superfamily. GDNF's ability to enhance the survival of dopaminergic neurons and other neuron populations demonstrates its therapeutic potential for the treatment of Parkinson's disease as well as other forms of nerve damage or malfunction.

The described biological activities of the neurturin neurotrophic factor include promoting the survival of nodose ganglia sensory neurons and a small population of dorsal root ganglia sensory neurons, in addition to superior cervical ganglion sympathetic neurons. The activity suggests the possibility of a common or similar signaling pathway. In addition, the biological activities of neurturin may extend to motor neurons and dopaminergic neurons. It has been demonstrated that receptors for GDNF and neurturin (described in U.S. Patent Application Serial No. 08/837,199 filed April 14, 1997 and WO 9740152, PCT/US97/6281) are structurally related.

The present invention is based upon the discovery of a novel protein product that binds to GDNF family receptor-alpha-3 (GFRα-3) which is described in U.S. Patent Application Serial No. 08/866,354 filed May 30, 1997 (PCT/US98/08486) the disclosure of which is incorporated by reference herein. The application provides the description of the cloning, expression and characterization of three GFR-α proteins. The receptor proteins include glial cell line-derived neurotrophic factor receptor-α and related receptor proteins 2 and 3 (GFRα-2 and GFRα-3).

In particular, the present invention involves the cloning, expression and characterization of a novel GDNF-related neurotrophic Factor. This molecule has been named GRNF4 because it is the fourth member of a group of structurally related proteins.

Nucleotide and amino acid sequences are described for GRNF4 protein products. A hydrophobic domain with the features of a signal peptide is found at the amino terminus. The GRNF4 gene encodes a secreted molecule of 224 amino acids that shares 30-40% homology with GDNF and neurturin. The mature form of GRNF4 is 106 amino acids long, and is 35% identical to GDNF and 46% identical to neurturin. GFRα-3 is a receptor of the GFRα family exclusively expressed in the peripheral sensory and sympathetic nervous systems. Binding and chemical crosslinking studies show that both soluble GFRα-3 and GFRα-3 expressed in cells bind the mature form GRNF4 efficiently and specifically. Binding of GRNF4 to GFRα-3 further induces the autophosphorylation or activation of the receptor protein tyrosine kinase Ret, indicating initiation of GRNF4 signaling. These data demonstrate that GRNF4 is a cognate ligand for GFRα-3 and its signaling may play a role in the development and/or maintenance of the peripheral sensory and autonomous nervous systems. Thus, GRNF4 is a potential therapeutic candidate for treating degenerative diseases of the peripheral nervous system, such as peripheral neuropathy.

The present invention enables the cloning of a GRNF4 protein product by providing a method for selecting target cells which express GRNF4. By providing a means of enriching for GRNF4-encoding nucleotide sequences, the present invention further provides for the purification of GRNF4 protein product and the direct cloning of GRNF4 -encoding DNA. The present description of the GRNF4 nucleotides and amino acid sequences provides the information needed to enable the reproduction of these entities as well as a variety of GRNF4 protein products. With this information, GRNF4 protein products may be isolated or generated by any means known to those skilled in the art of molecular synthesis, cloning and protein expression. A variety of means for the recombinant or synthetic production of GRNF4 molecules are disclosed.

As used herein, the term "GRNF4 protein" or "GRNF4 polypeptide" includes biologically active purified natural, synthetic or recombinant GRNF4 molecules, such as human and mouse GRNF4 as well as molecules which are at least 82% to 99.9% identical thereto as determined by one or more of the sequence comparison computer program algorithms, with their identified default parameters, as are well known in the art (e.g., the GAP, FASTA or BLAST programs as are discussed herein). Molecules within this 82-

100% identity range will include analogs or variants involving insertion, substitution and deletion variations (e.g., splice variants.) The term "GRNF4 protein product", as used herein, includes such GRNF4 proteins and polypeptides as well as chemically modified derivatives thereof.

The term "biologically active" or "GRNF4 activity", as used herein, refers to the binding of GRNF4 proteins and polypeptides (and protein products) to GFRα-3. In addition, the binding of the novel GRNF4 proteins and polypeptides to GFRα-3 induces tyrosine autophosphorylation or activation of the Ret receptor protein tyrosine kinase. Using the present disclosure, it is well within the ability of those of ordinary skill in the art to determine whether a GRNF4 protein product has a biological activity equivalent to that of the mouse and human GRNF4 molecules set forth in the Figures.

As used herein, the term "GRNF4 nucleic acids" or "GRNF4 polynucleotide" when used to describe a polynucleotide molecule refers to a polynucleotide molecule or fragment thereof that:

a) comprises a nucleotide sequence as set forth in Figures 2 or 6;

b) has a nucleotide sequence encoding a protein product comprising an amino acid sequence that is at least 77 percent identical to the protein product encoded by a polynucleotide sequence of mouse or human GRNF4 as described herein, but may have a sequence anywhere from 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90,91, 92, 93, 94, 95, 96, 97 or 98 to 99 percent identical to the protein product encoded by a polynucleotide sequence of mouse or human GRNF4 as described herein;

c) is a naturally occurring allelic variant or alternate splice variant of (a) or (b);

d) is a nucleic acid variant of (a)-(c) produced as provided for herein;

e) has a sequence that is complementary to (a)-(d);

f) hybridizes to any of (a)-(e) under conditions of high stringency and/or

g) has a nucleotide sequence encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or 25 amino acid substitutions, additions and/or deletions of any mature human GRNF4 protein product (i.e., an GRNF4 protein product with its endogenous signal peptide removed).

"Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between

polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. In accordance with the art, "identity" or "percent identical" as used herein measures the percent of identical matches between two or more sequences with gap alignments addressed by the particular algorithm. "Similarity" is a related concept, but in contrast to "identity", it measures both identical matches and conservative substitution matches. Therefore, in many cases, the degree of similarity between two polypeptide sequences will be higher than the percent identity between those two sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 19933; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods (i.e., "algorithms") to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux, J., et al., *Nucleic Acids Research* 12(1):387 (1984); Genetic Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol.* 215:403-410 (1990). The BLAST X program is publicly available from NCB and other sources (*BLAST Manual,* Altschul, S., et al. NCB NLM NIH Bethesda, MD 20894; Altschul, S., et al., *J. Mol. Biol.* 215:403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

By way of example, using the computer algorithm GAP (Genetic Computer Group, University of Wisconsin, Madison, WI), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty

(which is calculated as 3 X the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., in: Atlas of Protein Sequence and Structure, vol. 5, supp.3 [1978] for the PAM250 comparison matrix; see Hentikoff et al., Proc. Natl. Acad. Sci USA, 89:10915-10919 [1992] for the BLOSUM 62 comparison matrix) is also used by the algorithm.

The percent identity is then calculated by the algorithm by determining the percent identity as follows:

$$\frac{\text{Total number of identical matches in the matched span}}{\text{[length of the longer sequence within the matched span] + [number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Preferred parameters for polypeptide sequence comparison include the following:

Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970)

Comparison matrix: BLOSUM 62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps).

Preferred parameters for polynucleotide sequence comparison include the following:

Algorithm: Needleman and Wunsch, J. Mol Biol. 48:443-453 (1970)

Comparison matrix: matches = +10, mismatch = 0

Gap Penalty: 50

Gap Length Penalty: 3

The GAP program is also useful with the above parameters. The aforementioned parameters are the default parameters for polynucleotide comparisons.

Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, etc. may be used by those of skill in the art, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular choices to be made will depend on the specific comparison to be made, such as DNA to DNA, protein to protein, protein to DNA; and additionally, whether the comparison is between pairs of sequences (in which case GAP is generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

As demonstrated by a comparison of the mouse and human GRNF4 of the Figures, the sequences are 78.7% similar and 77.4% identical over the full length molecule. As demonstrated by the comparison of the amino acid sequences of mouse and human GRNF4 (Figure 8), the percent identity of mature forms of the molecule may be as high as 82 or 83 to 92% identical. Therefore, one skilled in the art will appreciate that a polynucleotide encoding a protein that has a 77%, or greater, identity as compared to human GRNF4 is recognized as a GRNF4 molecule. Protein products that are at least 82 percent identical (e.g., using the GAP program) will typically have several amino acid substitutions, deletions, and/or insertions as compared with any of the wild type GRNF4. Usually, the substitutions of the native residue will be either alanine, or a conservative amino acid so as to have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein. Possible substitutions are set forth in Table I.

Table I

Amino Acid Substitutions

Conservative:		
	Basic:	arginine
		lysine

	histidine
Acidic:	glutamic acid
	aspartic acid
Uncharged Polar:	glutamine
	asparagine
	serine
	threonine
	tyrosine
Non-Polar:	phenylalanine
	tryptophan
	cysteine
	glycine
	alanine
	valine
	proline
	methionine
	leucine
	isoleucine

Other preferred and exemplary substitutions by amino acid residue:

Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg

Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

The sources of GRNF4 protein products having high identities include the GRNF4 proteins or polypeptides of other mammals (such as depicted in the Figures) which are expected to have a high degree of identity to the human GRNF4 protein products. For example, the degree of homology between the mouse and human GRNF4 protein products disclosed herein is about 77%. GRNF4 proteins or polypeptides may be isolated from such mammals by virtue of cross-reactivity with antibodies to the mouse or human GRNF4 amino acid sequences depicted in the Figures. Alternatively, they may be expressed by polynucleotide molecules which are isolated through hybridization with the gene or with segments of the gene encoding the mouse or human GRNF4 molecules or which hybridize to a complementary sequence of the nucleotide sequences illustrated in the Figures.

The term "conditions of high stringency" refers to hybridization and washing under conditions that permit binding of a nucleic acid molecule used for screening, such as an oligonucleotide probe or cDNA molecule probe, to highly homologous sequences. An exemplary high stringency wash solution is 0.2 X SSC (saline-sodium citrate buffer) and 0.1 percent SDS (sodium dodecyl sulfate) used at a temperature of between 50°C-65°C. In general, to increase the stringency of a hybridization, lowering the salt concentration of the hybridization and wash solutions is favored over increasing the temperature.

Where oligonucleotide probes are used to screen cDNA or genomic libraries, one of the following two high stringency solutions may be used. The first of these is 6 X SSC

with 0.05 percent sodium pyrophosphate at a temperature of 35°C-62°C, depending on the length of the oligonucleotide probe. For example, 14 base pair probes are washed at 35-40°C, 17 base pair probes are washed at 45-50°C, 20 base pair probes are washed at 52-57°C, and 23 base pair probes are washed at 57-63°C. The temperature can be increased 2-3°C where the background non-specific binding appears high. A second high stringency solution utilizes tetramethylammonium chloride (TMAC) for washing oligonucleotide probes. One stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2 percent SDS. The washing temperature using this solution is a function of the length of the probe. For example, a 17 base pair probe is washed at about 45-50°C. Other suitable hybridization conditions are described in further detail below.

The novel GRNF4 protein products are typically isolated and purified to form materials which are substantially free of unwanted substances that would detract from the use of the present protein products for an intended purpose. For example, preferred GRNF4 protein products may be substantially free from the presence of other human (e.g., non-GRNF4) proteinaceous materials or pathological agents. Preferably, the GRNF4 protein products are about 80% free of other proteins which may be present due to the production technique used in the manufacture of the GRNF4 protein product. More preferably, the GRNF4 protein products are about 90% free of other proteins, particularly preferably, about 95% free of other proteins, and most preferably about ≥98% free of other proteins. In addition, the present invention furnishes the unique advantage of providing polynucleotide sequences for the manufacture of homogeneous GRNF4 protein products.

A variety of GRNF4 variants are contemplated, including addition, deletion and substitution variants. For example, a series of deletion variants may occur or may be made by removing one or more amino acid residues from the amino and/or carboxy termini of a GRNF4 molecule.

Using rules for the prediction of signal peptide cleavage as described by von Heijne (von Heijne, Nucleic Acids Research, 14, 4683-4690, 1986), the predicted cleavage of the signal peptide for mouse GRNF4 is between amino acids 39 and 40 (TEA-SL). The predicted cleavage of the signal peptide for human GRNF4 is between amino acids 47 and 48 (AEA-SL). The predicted mature forms of mouse and human GRNF4 are based on the

presence of Arg-Xaa-Xaa-Arg cleavage sites. Predicted mature forms include molecules having the following amino acid sequences:

mouse GRNF4	human GRNF4
amino acids 81-224	amino acids 81-228
amino acids 109-224	amino acids 89-228
amino acids 112-224	amino acids 113-228
amino acids 119-224	amino acids 116-228
amino acids 129-224	amino acids 133-224

Thus, it is contemplated that any or all of the residues from 1 through 80 to 1 through 132 may be removed from a GRNF4 without affecting binding to GFRα-3. Using known analysis techniques, it is further contemplated that C-terminal truncations may include the removal of one or more amino acid residues up to the last cysteine residue. Thus, GRNF4 protein products that are truncated forms of the molecule also may include the deletion of amino acid residues from either or both termini. Additional GRNF4 protein products are contemplated as involving a non-natural consensus sequence comprising as depicted in Figure 18 (SEQ ID NO:__), i.e., deleting, adding and/or substituting one or more amino acid residues to form consensus sequences, as based upon mouse and human GRNF4. Possible conservative, preferred and exemplary additions and substitutions are described above.

The present GRNF4 protein products and polynucleotides may be used for methods of treatment, or for methods of manufacturing medicaments for treatment. Such treatment includes the treatment of conditions responsive to the binding of GRNF4 to GFRα-3 and the activation of Ret receptor protein tyrosine kinase through GFRα-3.

Other aspects and advantages of the present invention will be apparent to those skilled in the art. For example, additional uses include new assay systems, transgenic animals and antibody production.

Study Models

The present invention provides for assay systems in which GRNF4 protein product

activity may be detected by measuring an elicited physiological response in a cell or cell line which expresses GFRα-3 and Ret. A physiological response may comprise a biological effect similar to that of GDNF or neurturin, including but not limited to, enhanced dopamine uptake, extension of neurites, increased cell survival or growth, as well as the transcriptional activation of certain nucleic acid sequences (e.g. promoter/enhancer elements as well as structural genes), GDNF-related processing, translation, or phosphorylation, and the induction of secondary processes in response to processes directly or indirectly induced by GDNF, to name but a few.

For example, a model system may be created which may be used to study the effects of excess GRNF4 activity. In such a system, the response of a cell to a GRNF4 protein product may be increased by engineering an increased number of suitable GFRα-3 and/or Ret on the cells of the model system relative to cells which have not been so modified. A system may also be developed to selectively provide an increased number of such GFRα-3s and/or Rets on cells which normally express GFRα-3 and/or Ret. In order to ensure expression of GFRα-3 and/or Ret, the GFRα-3 and/or Ret gene may be placed under the control of a suitable promoter sequence. It may be desirable to put the GFRα-3 gene under the control of a constitutive and/or tissue specific promoter (including but not limited to the CNS neuron specific enolase, neurofilament, and tyrosine hydroxylase promoter), an inducible promoter (such as the metallothionein promoter), the UV activated promoter in the human immunodeficiency virus long terminal repeat (Valeri et al., 1988, Nature 333:78-81), or the CMV promoter, or a developmentally regulated promoter.

By increasing the number of cellular GFRα-3s and/or Rets, the response to a GRNF4 protein product may be increased. If the model system contains little or no GRNF4 protein product, GRNF4 may be added to the system. It may also be desirable to add additional GRNF4 protein product to the model system in order to evaluate the effects of excess GRNF4 activity. Over expressing GRNF4 protein or polypeptide (or secreted GRNF4 protein or polypeptide) may be one method for studying the effects of elevated levels of GRNF4 on cells already expressing GFRα-3 and/or Ret.

GRNF4 Protein Product Therapies

In another aspect, certain conditions may benefit from an increase in GRNF4 level. This could be achieved through protein therapy or by cell or gene therapy, whereby selective expression of GRNF4 protein or polypeptide in appropriate cells is achieved, for example, by using GRNF4 genes controlled by tissue specific or inducible promoters or by producing localized infection with replication defective viruses carrying a recombinant GRNF4 gene.

It is envisioned that conditions which will benefit from GRNF4 protein product or combined GDNF or neurturin/GRNF4 protein product delivery include, but are not limited to, motor neuron disorders including amyotrophic lateral sclerosis, neurological disorders associated with diabetes, Parkinson's disease, Alzheimer's disease, and Huntington's chorea. Additional indications for the use of GRNF4 protein product or combined GDNF or neurturin/GRNF4 protein product delivery are described above and further include the treatment of: glaucoma or other diseases and conditions involving retinal ganglion cell degeneration; sensory neuropathy caused by injury to, insults to, or degeneration of, sensory neurons; sympathetic neuronal conditions; pathological conditions, such as inherited retinal degenerations and age, disease or injury-related retinopathies, in which photoreceptor degeneration occurs and is responsible for vision loss; and injury or degeneration of inner ear sensory cells, such as hair cells and auditory neurons for preventing and/or treating hearing loss due to variety of causes. In addition, it is envisioned that GRNF4 may be used in the treatment of peripheral sensory neuropathy or neurological disorders associated with improperly functioning peripheral sympathetic nerves. In yet another embodiment, it is contemplated that GRNF4 may be used to treat diseases associated with bone loss such as osteoporosis, osteogenesis imperfecta or hypercalcemia of malignancy. Furthermore, GRNF4 may be used to affect the development of osteoclasts, osteoblasts or chondrocytes.

Transgenic Animals

In yet another aspect, a recombinant GRNF4 gene may be used to inactivate or "knock out" the endogenous gene (e.g., by homologous recombination) and thereby create a GRNF4 deficient cell, tissue, or animal. For example, a recombinant GRNF4 gene may

be engineered to contain an insertional mutation which inactivates GRNF4. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by any conventional technique including transfection, transduction, injection, etc. Cells containing the construct may then be selected, for example by G418 resistance. Cells which lack an intact GRNF4 gene are then identified (e. g., by Southern blotting or Northern blotting or assay of expression). Cells lacking an intact GRNF4 gene may then be fused to early embryo cells to generate transgenic animals deficient in GRNF4. Such an animal may be used to define specific neuronal populations, or other in vivo processes, normally dependent upon GRNF4.

Diagnostic Applications

One variety of probe which may be used to detect GRNF4 expression is an oligonucleotide probe, which may be used to detect GRNF4-encoding RNA by any method known in the art, including, but not limited to, in situ hybridization, Northern blot analysis, or PCR related techniques. Nucleic acid products of the invention may be labeled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed in hybridization processes to locate the human GRNF4 gene position and/or the position of any related gene family in a chromosomal map. They may also be used for identifying human GRNF4 gene disorders at the DNA level and used as gene markers for identifying neighboring genes and their disorders. Contemplated herein are kits containing such labeled materials.

Protein products of the invention may be "labeled" by association with a detectable marker substance or label (e.g., a radioactive isotope, a fluorescent or chemiluminescent chemical, an enzyme or other label available to one skilled in the art) to provide reagents useful in detection and quantification of GFRα-3 in solid tissue and fluid samples such as blood or urine. Such products may also be used in detecting cells and tissues which are responsive to GRNF4 in normal or diseased states.

Another possible assay for detecting the presence or determining the level of GRNF4 in a test sample involves contacting the test sample with a GFRα-3 protein or anti-GNFR4 antibody, suitable for binding GRNF4, immobilized on a solid phase, thereby producing GFRα-3-bound or antibody-bound GRNF4. The GFRα-3-bound or antibody-

bound GRNF4 may optionally be contacted with a detection reagent, such as a labeled antibody specific for GRNF4, thereby forming a detectable product. Such assays may be developed in the form of assay devices for analyzing a test sample. In a basic form, such devices include a solid phase containing or coated with an appropriate GFRα-3 protein or anti-GRNF4 antibody.

The assay reagents provided herein may also be embodied as part of a kit or article of manufacture. Contemplated is an article of manufacture comprising a packaging material and one or more preparations of the presently provided polynucleotide or amino acid sequences. Such packaging material will comprise a label indicating that the preparation is useful for detecting GRNF4 in a biological sample. As such, the kit may optionally include materials to carry out such testing, such as reagents useful for performing protein analysis antibody binding, DNA or RNA hybridization analysis, or PCR analysis on blood, urine, or tissue samples.

Anti-GRNF4 Antibody

According to the present invention, GRNF4 protein products may be used as an immunogen to generate anti- GRNF4 antibodies. To further improve the likelihood of producing an anti- GRNF4 immune response, the amino acid sequence of GRNF4 may be analyzed in order to identify portions of the molecule which may be associated with increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes which present computer-generated plots of hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic helix, amphiphilic sheet, and secondary structure of GRNF4. Alternatively, the amino acid sequences of GRNF4 from different species could be compared, and relatively non-homologous regions identified; these non-homologous regions would be more likely to be immunogenic across various species.

Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within GRNF4, which fragments may possess one activity (e.g., immunological activity) and not others (e.g., GFRα-3 binding activity). Thus, the production of antibodies can include the production of anti-peptide antibodies.

Monoclonal antibodies directed against GRNF4 protein products may be prepared by any known technique which provides for the production of antibody molecules by continuous cell lines in culture. For example, the hybridoma technique originally developed by Kohler and Milstein to produce monoclonal antibodies (Nature, 256:495-497, 1975), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72, 1983), the EBV-hybridoma technique (Cole et al., in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96, 1985), and the like, may be used.

Human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies also may be prepared for therapeutic use and may be made by any of numerous techniques known in the art (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80:7308-7312, 1983; Kozbor et al., Immunology Today, 4:72-79, 1983; Olsson et al., Meth. Enzymol., 92:3-16, 1982). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81:6851, 1984; Takeda et al., Nature, 314:452, 1985).

Various procedures known in the art also may be used for the production of polyclonal antibodies. For the production of antibody, various host animals including, but not limited to, rabbits, mice, rats, etc., can be immunized by injection with GRNF4 polypeptide, or a fragment or derivative thereof. Various adjuvants may be used to increase the immunological response, depending on the host species selected. Useful adjuvants include, but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a GRNF4 epitope also may be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982) may be used to construct polynucleotide molecules which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as high performance liquid chromatography, or a combination thereof, etc. The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Such selective binding molecules may themselves be alternatives to GRNF4 protein products, and may be formulated as a pharmaceutical composition.

Recombinant Expression of GRNF4

The present invention provides various polynucleotides encoding GRNF4 protein products. The expression product or a derivative thereof is characterized by the ability to bind GDNF family receptor-alpha-3 (GFRα-3). The polynucleotides may also be used in cell therapy or gene therapy applications.

According to the present invention, novel GRNF4 protein products and DNA encoding all or part of such protein products are provided. Novel polynucleotide molecules of the invention are useful in securing expression in prokaryotic or eucaryotic host cells of molecules having at least a part of the primary structural conformation and one or more of the biological properties of recombinant human GRNF4. The polynucleotide molecules may be purified and isolated, so that the desired coding region is useful to produce the GRNF4 protein products. Alternatively, the nucleotide may be used for diagnostic purposes, as described more fully below. Exemplary DNA molecules of the present invention comprise nucleotides encoding GRNF4 polypeptides described herein. In addition, DNA molecules disclosed by the present invention include: (a) the GRNF4 DNA depicted in the Figures (and complementary strands); (b) a DNA which hybridizes (under hybridization conditions as disclosed herein, or equivalent conditions or more stringent conditions) to the DNA in subpart (a) or to fragments thereof; and (c) a DNA

which, but for the degeneracy of the genetic code, would hybridize to the DNA in subpart (a). Parts (b) and (c), above, may include genomic DNA encoding allelic variant forms of human GRNF4 and/or encoding GRNF4 from other mammalian species, and manufactured DNA sequences encoding GRNF4, fragments of GRNF4, and analogs of GRNF4 which DNA sequences may incorporate codons facilitating transcription and translation of messenger RNA in microbial hosts. Such manufactured sequences may readily be constructed according to the methods known in the art as well as the methods described herein.

Recombinant expression techniques, conducted in accordance with the descriptions set forth herein or other known methods, may be used to produce these polynucleotides and express the various GRNF4 protein products. For example, by inserting a DNA which encodes a GRNF4 protein product into an appropriate vector, one skilled in the art can readily produce large quantities of the desired nucleotide. The sequences can then be used to generate detection probes or amplification primers. Alternatively, a polynucleotide encoding a GRNF4 can be inserted into an expression vector. By introducing the expression vector into an appropriate host, the desired GRNF4 protein product may be produced in large amounts.

As further described herein, there are numerous host/vector systems available for the propagation of DNA and/or the production of GRNF4 protein products. These include, but are not limited to, plasmid, viral and insertional vectors, and prokaryotic and eukaryotic hosts. One skilled in the art can adapt a host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

By means of such recombinant techniques, the GRNF4 protein products of the present invention are readily produced in commercial quantities with greater purity. Furthermore, it will be appreciated by those skilled in the art that the present disclosure provides novel nucleotides including degenerate nucleotides encoding the GRNF4 protein products specifically set forth in the Figures, as well as sequences encoding variants or analogs of GRNF4 protein products, and those nucleotides which hybridize, preferably under stringent hybridization conditions, to complements of these DNA molecules (see, Maniatis et. al., Molecular Cloning (A Laboratory Manual); Cold Spring Harbor

Laboratory, pages 387 to 389, 1982.) Exemplary stringent hybridization conditions include hybridization in 4 x SSC at 62-67°C, followed by washing in 0.1 x SSC at 62-67°C for approximately an hour. Alternatively, exemplary stringent hybridization conditions include hybridization in 45-55% formamide, 4 x SSC at 40-45°C. It will be further appreciated by those skilled in the art that the present invention also provides for DNA which hybridize to the complementary sequences of mouse and human GRNF4 under relaxed and stringent hybridization conditions and which encode a protein product having GRNF4 activity. Examples of such relaxed stringency hybridization conditions are 4 x SSC at 45-55°C or hybridization with 30-40% formamide at 40-45°C.

<u>Preparation of Polynucleotides Encoding GRNF4</u>

Based upon the disclosure of the present invention, a nucleotide encoding a full length GRNF4 protein product or a fragment thereof may readily be prepared or obtained by a variety of means, including, without limitation, chemical synthesis, cDNA or genomic library screening, expression library screening, and/or PCR amplification of cDNA. These methods and others useful for preparing DNA are known in the art and are set forth, for example, by Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), by Ausubel et al., eds (Current Protocols in Molecular Biology, Current Protocols Press, 1994), and by Berger and Kimmel (Methods in Enzymology: Guide to Molecular Cloning Techniques, vol. 152, Academic Press, Inc., San Diego, CA, 1987). Preferred polynucleotide molecules encoding GRNF4 protein products are mammalian sequences.

Chemical synthesis of a DNA which encodes a GRNF4 protein product can also be accomplished using methods known in the art, such as those set forth by Engels et al. (Angew. Chem. Intl. Ed., 28:716-734, 1989). These methods include, inter alia, the phosphotriester, phosphoramidite and H-phosphonate methods of nucleic acid sequence synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry. Typically, the DNA encoding the desired protein product will be several hundred base pairs (bp) or nucleotides in length. Nucleic acid sequences larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated together to form a sequence for

the expression of a full length GRNF4 protein product or a portion thereof.

Alternatively, a suitable DNA may be obtained by screening an appropriate cDNA library (i.e., a library prepared from one or more tissue source(s) believed to express the protein) or a genomic library (a library prepared from total genomic DNA). The source of the cDNA library is typically a tissue that has been found or is believed to express GRNF4 in reasonable quantities. Typically, the source of the genomic library is any tissue or tissues from a mammalian species believed to harbor a gene encoding GRNF4. The library can be screened for the presence of the GRNF4 cDNA/gene using one or more probes (such as oligonucleotides, cDNA or genomic DNA fragments based upon the presently disclosed sequences) that will hybridize selectively with GRNF4 cDNA(s) or gene(s) present in the library. The probes typically used for such library screening usually encode a small region of GRNF4 DNA from the same or a similar species as the species from which the library was prepared. Alternatively, the probes may be degenerate, as discussed herein.

Library screening is typically accomplished by annealing the oligonucleotide probe or cDNA to the clones in the library under conditions of stringency that prevent non-specific binding but permit binding (hybridization) of those clones that have a significant level of identity with the probe or primer. Typical hybridization and washing stringency conditions depend in part on the size (i.e., number of nucleotides in length) of the cDNA or oligonucleotide probe, and whether the probe is degenerate. The probability of obtaining a clone(s) is also considered in designing the hybridization solution (e.g., whether a cDNA or genomic library is being screened; if it is a cDNA library, the probability that the cDNA of interest is present at a high level).

Where DNA fragments (such as cDNAs) are used as probes, typical hybridization conditions include those as set forth in Ausubel et al., eds., supra. After hybridization, the blot containing the library is washed at a suitable stringency, depending on several factors such as probe size, expected identity of probe to clone, type of library being screened, number of clones being screened, and the like. Examples of stringent washing solutions (which are usually low in ionic strength and are used at relatively high temperatures) are as follows. One such stringent wash is 0.015 M NaCl, 0.005 M NaCitrate and 0.1% SDS at 55-65°C. Another such stringent buffer is 1 mM Na_2EDTA, 40 mM $NaHPO_4$, pH 7.2,

and 1% SDS at about 40-50°C. Yet another stringent wash is 0.2 X SSC and 0.1% SDS at about 50-65°C.

There are also exemplary protocols for stringent washing conditions where oligonucleotide probes are used to screen cDNA or genomic libraries. For example, a first protocol uses 6 X SSC with 0.05 percent sodium pyrophosphate at a temperature of between about 35 and 62°C, depending on the length of the probe. For example, 14 base probes are washed at 35-40°C, 17 base probes at 45-50°C, 20 base probes at 52-57°C, and 23 base probes at 57-63°C. The temperature can be increased 2-3°C where the background non-specific binding appears high. A second protocol uses tetramethylammonium chloride (TMAC) for washing. One such stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2% SDS.

Another suitable method for obtaining a polynucleotide molecule encoding a GRNF4 protein product is by polymerase chain reaction (PCR). In this method, poly(A)+RNA or total RNA is extracted from a tissue that expresses GRNF4. A cDNA is then prepared from the RNA using the enzyme reverse transcriptase (i.e., RT-PCR). Two primers, typically complementary to two separate regions of the GRNF4 cDNA (oligonucleotides), are then added to the cDNA along with a polymerase such as Taq polymerase, and the polymerase amplifies the cDNA region between the two primers.

Where the method of choice for preparing the DNA encoding the desired GRNF4 protein product requires the use of oligonucleotide primers or probes (e.g., PCR, cDNA or genomic library screening), the oligonucleotide sequences selected as probes or primers should be of adequate length and sufficiently unambiguous so as to minimize the amount of non-specific binding that will occur during library screening or PCR amplification. The actual sequence of the probes or primers is usually based on conserved or highly identical or homologous sequences or regions from the same or a similar gene from another organism, such as the mouse polynucleotide molecule involved in the present invention. Optionally, the probes or primers can be fully or partially degenerate, i.e., contain a mixture of probes/primers, all encoding the same amino acid sequence, but using different codons to do so. An alternative to preparing degenerate probes is to place an inosine in some or all of those codon positions that vary by species. The oligonucleotide probes or primers may be prepared by chemical synthesis methods for DNA as described above.

GRNF4 protein products based on these DNA encoding GRNF4, including mutant or variant sequences, are also contemplated by the present invention. Mutant or variant sequences include those sequences containing one or more nucleotide substitutions, deletions, and/or insertions as compared to the wild type sequence and that result in the expression of amino acid sequence variations as compared to the wild type amino acid sequence. In some cases, naturally occurring GRNF4 amino acid mutants or variants may exist, due to the existence of natural allelic variation. GRNF4 protein products based on such naturally occurring mutants or variants are envisioned by the present invention. Preparation of synthetic mutant sequences is also well known in the art, and is described for example in Wells et al. (Gene, 34:315, 1985) and in Sambrook et al., supra.

In some cases, it may be desirable to prepare variants of naturally occurring GRNF4 proteins or polypeptides. Nucleotide variants (wherein one or more nucleotides are designed to differ from the wild-type or naturally occurring GRNF4) may be produced using site directed mutagenesis or PCR amplification where the primer(s) have the desired point mutations (see Sambrook et al., supra, and Ausubel et al., supra, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels et al., supra, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well. Preferred DNA variants are those containing nucleotide substitutions accounting for codon preference in the host cell that is to be used to recombinantly produce GRNF4. Other preferred variants are those encoding conservative amino acid changes (e.g., wherein the charge or polarity of the naturally occurring amino acid side chain is not altered substantially by substitution with a different amino acid) as compared to wild type, and/or those designed to either generate a novel glycosylation and/or phosphorylation site(s) on GRNF4, or those designed to delete an existing glycosylation and/or phosphorylation site(s) on GRNF4. Yet other preferred variants are those DNA encoding a GRNF4 protein product based upon a GRNF4 consensus sequence as depicted in the Figures.

Vectors

The cDNA or genomic DNA encoding the desired GRNF4 protein product is inserted into a vector for further cloning (amplification of the DNA) and/or for expression.

Suitable vectors are commercially available, or the vector may be specially constructed. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to plasmids such as Bluescript® plasmid derivatives (a high copy number ColE1-based phagemid, Stratagene Cloning Systems Inc., La Jolla CA), PCR cloning plasmids designed for cloning Taq-amplified PCR products (e.g., TOPO™ TA Cloning® Kit, PCR2.1® plasmid derivatives, Invitrogen, Carlsbad, CA), and mammalian , yeast or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, CA). The recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, or other known techniques.

For example, the DNA encoding GRNF4 is inserted into a cloning vector which is used to transform, transfect, or infect appropriate host cells so that many copies of the nucleotide are generated. This can be accomplished by ligating a DNA fragment into a cloning vector which has complementary cohesive termini. If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. It also may prove advantageous to incorporate restriction endonuclease cleavage sites into the oligonucleotide primers used in polymerase chain reaction to facilitate insertion of the resulting polynucleotide sequence into vectors. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and DNA encoding GRNF4 may be modified by homopolymeric tailing. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated GRNF4 gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the GRNF4-encoding nucleotide may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The selection or construction of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be

inserted into the vector, and 3) the host cell (e.g., mammalian, insect, yeast, fungal, plant or bacterial cells) to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and its compatibility with the intended host cell. For DNA expression, the vector components may include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selection or marker genes, enhancer elements, promoters, a transcription termination sequence, and the like. These components may be obtained from natural sources or synthesized by known procedures. The vectors of the present invention involve a DNA which encodes the GRNF4 protein product of interest operatively linked to one or more amplification, expression control, regulatory or similar operational elements capable of directing, controlling or otherwise effecting the amplification or expression of the DNA in the selected host cell.

Expression vectors containing GRNF4 DNA inserts can be identified by three general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of "marker" gene functions, and (c) the expression of inserted sequences. In the first approach, the presence of a foreign nucleotide sequence inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted GRNF4-encoding molecule. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a foreign nucleotide into the vector. For example, if a GRNF4-encoding sequence is inserted within the marker gene sequence of the vector, recombinants containing the GRNF4 insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by detecting the foreign molecule expressed by the recombinant nucleotide. Such assays can be based on the physical or functional properties of the expressed GRNF4 protein product, for example, by binding of the GRNF4 protein product to an antibody which directly recognizes GRNF4.

Signal Sequence

The signal sequence may be a component of the vector, or it may be a part of GRNF4 DNA that is inserted into the vector. The native GRNF4 DNA encodes a signal sequence at the amino terminus of the protein that is cleaved during post-translational processing of the molecule to form the mature GRNF4 protein product. Included within the scope of this invention are GRNF4 nucleotides with the native signal sequence as well as GRNF4 nucleotides wherein the native signal sequence is deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native GRNF4 signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders. For yeast secretion, the native GRNF4 signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

Origin of Replication

Expression and cloning vectors generally include a polynucleotide molecule that enables the vector to replicate in one or more selected host cells. In cloning vectors, this sequence is typically one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeasts, and viruses. The origin of replication from the plasmid pBR322 (Product No. 303-3S, New England Biolabs, Beverly, MA) is suitable for most Gram-negative bacteria and various origins (e.g., SV40, polyoma, adenovirus, vesicular stomatitus virus (VSV) or papillomaviruses such as HPV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

Selection Gene

The expression and cloning vectors may contain a selection gene. This gene encodes a "marker" protein necessary for the survival or growth of the transformed host cells when grown in a selective culture medium. Host cells that were not transformed with the vector will not contain the selection gene, and therefore, they will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from the culture medium.

Other selection genes may be used to amplify the gene which will be expressed. Amplification is the process wherein genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the marker present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes GRNF4. As a result, increased quantities of GRNF4 are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate, a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is used is the Chinese hamster ovary cell line deficient in DHFR activity (see, for example, Urlaub and Chasin, Proc. Natl. Acad. Sci., U.S.A., 77(7): 4216-4220, 1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA present in the expression vector, such as the DNA encoding a GRNF4 protein product.

Promoter

The expression and cloning vectors of the present invention will typically contain a promoter that is recognized by the host organism and operably linked to the molecule encoding the GRNF4 protein product. Promoters are untranslated sequences located upstream(5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular molecule, such as that encoding GRNF4. Promoters are conventionally grouped into one of two classes, inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. A large number of promoters, recognized by a variety of potential host cells, are well known. These promoters are operably linked to the DNA encoding GRNF4 by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence into the vector. The native GRNF4 promoter sequence may be used to direct amplification and/or expression of GRNF4 DNA. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein as compared to the native promoter, and if it is compatible with the host cell system that has been selected for use.

Promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase, a tryptophan (trp) promoter system; and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their sequences have been published, thereby enabling one skilled in the art to ligate them to the desired DNA sequence(s), using linkers or adapters as needed to supply any required restriction sites.

Suitable promoting sequences for use with yeast hosts are also well known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the

actin promoter.

Additional promoters which may be of interest in controlling GRNF4 expression include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, Nature, 290:304-310, 1981); the CMV promoter; the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., Cell, 22:787-797, 1980); the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A., 78:144-1445, 1981); the regulatory sequences of the metallothionine gene (Brinster et al., Nature, 296:39-42, 1982); prokaryotic expression vectors such as the beta -lactamase promoter (Villa-Kamaroff, et al., Proc. Natl. Acad. Sci. U.S.A., 75:3727-3731, 1978); or the tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. U.S.A., 80:21-25, 1983). Also of interest are the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: the elastase I gene control region which is active in pancreatic acinar cells (Swift et al., Cell, 38:639-646, 1984; Ornitz et al., Cold Spring Harbor Symp. Quant. Biol. 50:399-409, 1986; MacDonald, Hepatology, 7:425-515, 1987); the insulin gene control region which is active in pancreatic beta cells (Hanahan, Nature, 315:115-122, 1985); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., Cell, 38:647-658, 1984; Adames et al., Nature, 318:533-538, 1985; Alexander et al., Mol. Cell. Biol., 7:1436-1444, 1987); the mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., Cell, 45:485-495, 1986), albumin gene control region which is active in liver (Pinkert et al., Genes and Devel., 1:268-276, 1987); the alphafetoprotein gene control region which is active in liver (Krumlauf et al., Mol. Cell. Biol., 5:1639-1648, 1985; Hammer et al., Science, 235:53-58, 1987); the alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., Genes and Devel., 1:161-171, 1987); the beta-globin gene control region which is active in myeloid cells (Mogram et al., Nature, 315:338-340, 1985; Kollias et al., Cell, 46:89-94, 1986); the myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., Cell, 48:703-712, 1987); the myosin light chain-2 gene control region which is active in skeletal muscle (Sani, Nature, 314:283-286, 1985); and the gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., Science, 234:1372-1378, 1986).

Enhancer Element

An enhancer sequence may be inserted into the vector to increase the transcription of a DNA encoding a GRNF4 protein product of the present invention by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase its transcription. Enhancers are relatively orientation and position independent. They have been found 5' and 3' to the transcription unit. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus will be used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be spliced into the vector at a position 5' or 3' to GRNF4 DNA, it is typically located at a site 5' from the promoter.

Transcription Termination

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for terminating transcription and stabilizing the mRNA. Such sequences are commonly available from the 5' and occasionally 3' untranslated regions of eukaryotic DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding GRNF4 protein products.

The construction of suitable vectors containing one or more of the above-listed components together with the desired GRNF4-encoding molecule is accomplished by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the desired order to generate the plasmids required. To confirm that the correct sequences have been constructed, the ligation mixtures may be used to transform E. coli, and successful transformants may be selected by known techniques, such as ampicillin or tetracycline resistance as described above. Plasmids from the transformants may then be prepared, analyzed by restriction endonuclease digestion, and/or sequenced to

confirm the presence of the desired construct.

Vectors that provide for the transient expression of DNA encoding GRNF4 protein products in mammalian cells may also be used. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of the desired protein encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of proteins encoded by cloned DNAs, as well as for the rapid screening of such proteins for desired biological or physiological properties. Thus, transient expression systems are particularly useful in identifying variants of the protein.

Selection and Transformation of Host Cells

Host cells (e.g., bacterial, mammalian, insect, yeast, or plant cells) transformed with polynucleotide molecules for use in expressing recombinant GRNF4 protein products are also provided by the present invention. The transformed host cell is cultured under appropriate conditions permitting the expression of the nucleotide sequence. The selection of suitable host cells and methods for transformation, culture, amplification, screening and product production and purification are well known in the art. See for example, Gething and Sambrook, Nature, 293: 620-625 (1981), or alternatively, Kaufman et al., Mol. Cell. Biol., 5 (7): 1750-1759 (1985) or Howley et al., U.S. Pat. No. 4,419,446. Additional exemplary materials and methods are discussed herein. The transformed host cell is cultured in a suitable medium, and the expressed GRNF4 protein product is then optionally recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by an appropriate means known to those skilled in the art.

Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein or polypeptide. Expression in yeast may be used to produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of the

heterologous GRNF4 protein or polypeptide. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

Suitable host cells for cloning or expressing the vectors disclosed herein are prokaryote, yeast, or higher eukaryote cells. Eukaryotic microbes such as filamentous fungi or yeast may be suitable hosts for the expression of GRNF4 protein products. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms, but a number of other genera, species, and strains are well known and commonly available.

Host cells to be used for the expression of glycosylated GRNF4 protein products are also derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture might be used, whether such culture involves vertebrate or invertebrate cells, including plant and insect cells. The propagation of vertebrate cells in culture (tissue culture) is a well known procedure. Examples of useful mammalian host cell lines include, but are not limited to, monkey kidney CV1 line transformed by SV40 (COS7), human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), baby hamster kidney cells, and Chinese hamster ovary cells. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines (American Type Culture Collection (ATCC), Rockville, MD). Each of these cell lines is known by and available to those skilled in the art of protein expression.

Suitable host cells also include prokaryotic cells. Prokaryotic host cells include, but are not limited to, bacterial cells, such as Gram-negative or Gram-positive organisms, for example, E. coli, Bacilli such as B. subtilis, Pseudomonas species such as P. aeruginosa, Salmonella typhimurium, or Serratia marcescans. For example, the various strains of E. coli (e.g., HB101, DH5a, DH10, XL-1 blue and MC1061) (Clontech, Palo Alto, CA and Stratagene, La Jolla, CA) are well-known as host cells in the field of biotechnology. Various strains of Streptomyces spp. and the like may also be employed. Presently preferred host cells for producing GRNF4 protein products are bacterial cells (e.g., Escherichia coli) and mammalian cells (such as Chinese hamster ovary cells, COS

cells, etc.)

The host cells are transfected and preferably transformed with the above-described expression or cloning vectors and cultured in a conventional nutrient medium. The medium may be modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transfection and transformation are performed using standard techniques which are well known to those skilled in the art and which are selected as appropriate to the host cell involved. For example, for mammalian cells without cell walls, the calcium phosphate precipitation method may be used. Electroporation, micro injection and other known techniques may also be used.

Culturing the Host Cells

Transformed cells used to produce GRNF4 protein products of the present invention are cultured in suitable media. The media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as gentamicin), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or other energy source. Other supplements may also be included, at appropriate concentrations, as will be appreciated by those skilled in the art. Suitable culture conditions, such as temperature, pH, and the like, are also well known to those skilled in the art for use with the selected host cells.

Once the GRNF4 protein product is produced, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. For example, a GRNF4 protein product may be isolated by binding to an affinity column comprising anti-GRNF4 antibody bound to a stationary support.

Homologous Recombination

It is further envisioned that GRNF4 protein products may be produced by homologous recombination, or with recombinant production methods utilizing control

elements introduced into cells already containing DNA encoding GRNF4. For example, homologous recombination methods may be used to modify a cell that contains a normally transcriptionally silent GRNF4 gene, or under expressed gene, and thereby produce a cell which expresses GRNF4. Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally active genes (Kucherlapati, Prog. in Nucl. Acid Res. and Mol. Biol., 36:301, 1989). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al., Cell, 44:419-428, 1986; Thomas and Capecchi, Cell, 51:503-512, 1987; Doetschman et al., Proc. Natl. Acad. Sci., 85:8583-8587, 1988) or to correct specific mutations within defective genes (Doetschman et al., Nature, 330:576-578, 1987). Exemplary homologous recombination techniques are described in U.S. 5,272,071 (EP 91 90 3051, EP Publication No. 505 500; PCT/US90/07642, International Publication No. WO 91/09955) the disclosure of which is hereby incorporated by reference.

Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by attaching it to targeting DNA. The targeting DNA is DNA that is complementary (homologous) to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the DNA replication process. It is a general property of DNA that has been inserted into a cell to hybridize, and therefore, recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an oligonucleotide that contains a mutation or a different sequence of DNA, it too is incorporated into the newly synthesized strand as a result of the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transferred DNA is incorporated into the genome.

If the sequence of a particular gene is known, such as the nucleotide sequence of GRNF4 presented herein, a piece of DNA that is complementary to a selected region of the gene can be synthesized or otherwise obtained, such as by appropriate restriction of the native DNA at specific recognition sites bounding the region of interest. This piece serves as a targeting sequence upon insertion into the cell and will hybridize to its homologous

region within the genome. If this hybridization occurs during DNA replication, this piece of DNA, and any additional sequence attached thereto, will act as an Okazaki fragment and will be backstitched into the newly synthesized daughter strand of DNA. The present invention, therefore, includes nucleotide sequences encoding a GRNF4 molecule, which nucleotides may be used as targeting sequences.

Attached to these pieces of targeting DNA are regions of DNA which may interact with the expression of a GRNF4 protein product. For example, a promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired GRNF4 protein product. The control element does not encode GRNF4, but instead controls a portion of the DNA present in the host cell genome. Thus, the expression of GRNF4 protein products may be achieved not by transfection of DNA that encodes the GRNF4 gene itself, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest) coupled with DNA regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of a GRNF4 protein product.

A. GRNF4 Variants

As discussed above, the GRNF4 protein products disclosed herein include molecules in which amino acids have been deleted from ("deletion variants"), inserted into ("addition variants"), or substituted for ("substitution variants") residues within the amino acid sequence of naturally-occurring GRNF4 including those depicted in the Figures. Such variants are prepared by introducing appropriate nucleotide changes into the DNA encoding the GRNF4 molecule or by in vitro chemical synthesis of the desired protein or polypeptide. It will be appreciated by those skilled in the art that many combinations of deletions, insertions, and substitutions can be made to an amino acid sequence such as mature human GRNF4 provided that the final molecule possesses GRNF4 activity.

Based upon the present description of particular GRNF4 amino acid sequences from multiple species, as well as the consensus sequences derived therefrom, one can readily design and manufacture a variety of nucleotides suitable for use in the recombinant (e.g., microbial) expression of protein products having primary conformations which differ

from those depicted in the Figures in terms of the identity or location of one or more residues. Mutagenesis techniques for the replacement, insertion or deletion of one or more selected amino acid residues encoded by the nucleotides are well known to one skilled in the art (e.g., U.S. Pat. No. 4,518,584, the disclosure of which is hereby incorporated by reference.) There are two principal variables in the construction of substitution variants: the location of the mutation site and the nature of the mutation. In designing GRNF4 substitution variants, the selection of the mutation site and nature of the mutation will depend on the GRNF4 characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid modifications and then with more radical selections depending upon the results achieved, (2) deleting the target amino acid residue, or (3) inserting amino acid residues adjacent to the located site. Conservative changes in from 1 to 8 contiguous amino acids are preferred. N-terminal and C-terminal deletion GRNF4 variants may also be generated by proteolytic enzymes.

For GRNF4 deletion variants, deletions generally range from about 1 to 8 contiguous residues, more usually from about 1 to 4 contiguous residues, and typically from about 1 to 2 contiguous residues. N-terminal, C-terminal and internal intrasequence deletions are contemplated. Deletions may be introduced into regions of the molecule which have low identity with non-human GRNF4 to modify the activity of GRNF4. Deletions in areas of substantial homology with non-human GRNF4 sequences will be more likely to significantly modify GRNF4 biological activity. The number of consecutive deletions typically will be selected so as to preserve the tertiary structure of the GRNF4 protein product in the affected domain, e.g., cysteine crosslinking. Non-limiting examples of deletion variants include truncated GRNF4 molecules which lack N-terminal or C-terminal amino acid residues.

For GRNF4 addition variants, amino acid sequence additions typically include N- and/or C-terminal fusions or terminal additions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as internal or medial additions of single or multiple amino acid residues. Protein products of the invention may also include an initial methionine amino acid residue (at position -1 with respect to the first amino acid residue of the desired polypeptide). Internal additions may range generally

from about 1 to 8 contiguous residues, more typically from about 1 to 4 residues, and usually from about 1 to 2 amino acid residues. Examples of N-terminal addition variants include GRNF4 with the inclusion of a heterologous N-terminal signal sequence to the N-terminus of GRNF4 to facilitate the secretion of mature GRNF4 from recombinant host cells and thereby facilitate harvesting or bioavailability. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Additions may also include amino acid sequences derived from the sequence of other neurotrophic factors. For example, it is contemplated that a fusion protein of GDNF and GRNF4 or neurturin and GRNF4, may be produced, with or without a linking sequence, thereby forming a single molecule therapeutic entity.

GRNF4 substitution variants have one or more amino acid residues of the GRNF4 amino acid sequence removed and a different residue(s) inserted in its place. Such substitution variants include allelic variants, which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. As with the other variant forms, substitution variants may involve the replacement of single or contiguous amino acid residues at one or more different locations.

Specific mutations of the GRNF4 amino acid sequence may involve modifications to a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of an O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) result in non-glycosylation at the modified tripeptide sequence. Thus, the expression of appropriate altered nucleotide sequences produces variants which are not glycosylated at that site. Alternatively, the GRNF4 amino acid sequence may be modified to add glycosylation sites.

One method for identifying GRNF4 amino acid residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244: 1081-1085, 1989). In this method, an amino acid residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions may then be refined by introducing additional or alternate residues at the sites of substitution. Thus, the target site for introducing an amino acid sequence variation is determined, alanine scanning or random mutagenesis is conducted on the corresponding target codon or region of the DNA sequence, and the expressed GRNF4 variants are screened for the optimal combination of desired activity and degree of activity.

Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleotides) are expected to produce GRNF4 protein products having functional and chemical characteristics similar to those of naturally occurring GRNF4. In contrast, substantial modifications in the functional and/or chemical characteristics of GRNF4 protein products may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues may be divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr;
3) acidic: Asp, Glu;
4) basic: Asn, Gln, His, Lys, Arg;
5) residues that influence chain orientation: Gly, Pro; and
6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of the human GRNF4 molecule that are homologous with non-

human GRNF4, or into the non-homologous regions of the molecule.

Thus, it is envisioned that GRNF4 protein products will include those biologically active molecules containing all or part of the amino acid sequences as depicted in the Figures, as well as consensus and modified sequences in which amino acid residues substitutions result in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It is also contemplated that the GRNF4 protein products may be differentially modified during or after translation, e.g., by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand.

B. GRNF4 Derivatives

Chemically modified derivatives of GRNF4 or GRNF4 analogs may be prepared by one of skill in the art based upon the present disclosure. The chemical moieties most suitable for derivatization include water soluble polymers. A water soluble polymer is desirable because the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. The effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (e.g., by osmotic pump, or, more preferably, by injection or infusion, or, further formulated for oral, pulmonary or other

delivery routes), and determining its effectiveness.

Suitable water soluble polymers include, but are not limited to, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2 kDa and about 100 kDa for ease in handling and manufacturing (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired; the effects, if any, on biological activity; the ease in handling; the degree or lack of antigenicity and other known effects of polyethylene glycol on a therapeutic protein or variant).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or polypeptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art.

See for example, EP 0 401 384, the disclosure of which is hereby incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol., 20: 1028-1035, 1992 (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydrl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). For therapeutic purposes, attachment at an amino group, such as attachment at the N-terminus or lysine group is preferred. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire an N-terminal chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the e-amino group of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the

polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

The present invention contemplates use of derivatives which are prokaryote-expressed GRNF4 linked to at least one polyethylene glycol molecule, as well as use of GRNF4 attached to one or more polyethylene glycol molecules via an acyl or alkyl linkage.

Pegylation may be carried out by any of the pegylation reactions known in the art. See, for example: Focus on Growth Factors, 3 (2): 4-10, 1992; EP 0 154 316, the disclosure of which is hereby incorporated by reference; EP 0 401 384; and the other publications cited herein that relate to pegylation. The pegylation may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer).

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with the GRNF4 molecule. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of GRNF4. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide (NHS). As used herein, "acylation" is contemplated to include without limitation the following types of linkages between the therapeutic protein and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like. See Bioconjugate Chem., 5: 133-140, 1994. Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions such as temperature, solvent, and pH that would inactivate the GRNF4 protein or polypeptide to be modified.

Pegylation by acylation will generally result in a poly-pegylated GRNF4 protein product. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., > 95%) mono, di- or tri-pegylated. However, some species with higher degrees of pegylation may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard

purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with GRNF4 in the presence of a reducing agent. Pegylation by alkylation can also result in poly-pegylated GRNF4 protein products. In addition, one can manipulate the reaction conditions to favor pegylation substantially only at the a-amino group of the N-terminus of GRNF4 (i.e., a mono-pegylated protein). In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a $-CH_2-NH-$ group. With particular reference to the $-CH_2-$ group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization. The reaction is performed at a pH which allows one to take advantage of the pKa differences between the e-amino groups of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. In one important aspect, the present invention contemplates use of a substantially homogeneous preparation of monopolymer/GRNF4 conjugate molecules (meaning a GRNF4 protein or polypeptide to which a polymer molecule has been attached substantially only (i.e., > 95%) in a single location). More specifically, if polyethylene glycol is used, the present invention also encompasses use of pegylated GRNF4 proteins or polypeptides lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the GRNF4 protein or polypeptide.

Thus, GRNF4 protein products according to the present invention include pegylated GRNF4, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, and preferably 2-5, PEG groups). The PEG groups are generally attached to the protein at the a- or e-amino groups of amino acids, but it is also contemplated that the PEG

groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

The polymer molecules used in both the acylation and alkylation approaches may be selected from among water soluble polymers as described above. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, preferably, so that the degree of polymerization may be controlled as provided for in the present methods. An exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see, U.S. Patent 5,252,714). The polymer may be branched or unbranched. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally-occurring glycosyl residues since these are usually made more conveniently by mammalian recombinant expression systems. The polymer may be of any molecular weight, and may be branched or unbranched.

An exemplary water-soluble polymer for use herein is polyethylene glycol. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be performed under any suitable condition used to react a biologically active substance with an activated polymer molecule. Methods for preparing a pegylated GRNF4 will generally comprise the steps of (a) reacting a GRNF4 protein or polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the molecule becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of PEG:protein, the greater the percentage of poly-pegylated product.

Reductive alkylation to produce a substantially homogeneous population of mono-polymer/GRNF4 will generally comprise the steps of: (a) reacting a GRNF4 protein or polypeptide with a reactive PEG molecule under reductive alkylation conditions, at a pH

suitable to permit selective modification of the a-amino group at the amino terminus of GRNF4; and (b) obtaining the reaction product(s).

For a substantially homogeneous population of mono-polymer/GRNF4, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of GRNF4. Such reaction conditions generally provide for pKa differences between the lysine amino groups and the a-amino group at the N-terminus (the pKa being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal a-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-9, preferably 3-6.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer polymer molecules may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2 kDa to about 100 kDa. The preferred average molecular weight is about 5 kDa to about 50 kDa, particularly preferably about 12 kDa to about 25 kDa. The ratio of water-soluble polymer to GRNF4 will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any GRNF4 molecule having an a-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/GRNF4 conjugate. The term "monopolymer/GRNF4 conjugate" is used here to mean a composition comprised of a single polymer molecule attached to a molecule of GRNF4. The monopolymer/GRNF4 conjugate typically will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation

will generally be greater than 90% monopolymer/GRNF4 conjugate, and more usually greater than 95% monopolymer/GRNF4 conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety). It is also envisioned that the GRNF4 protein product may involve the preparation of a pegylated molecule involving a fusion protein or linked GRNF4 and neurotrophic factor, such as GRNF4 and GDNF molecules or GRNF4 and neurturin molecules.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Suitable reducing agents may be selected from sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly suitable reducing agent is sodium cyanoborohydride. Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined case-by-case based on the published information relating to derivatization of proteins with water soluble polymers (see the publications cited herein).

C. GRNF4 Pharmaceutical Compositions

GRNF4 protein product pharmaceutical compositions typically include a therapeutically or prophylactically effective amount of GRNF4 protein product in admixture with one or more pharmaceutically and physiologically acceptable formulation materials selected for suitability with the mode of administration. Suitable formulation materials include, but are not limited to, antioxidants, preservatives, coloring, flavoring and diluting agents, emulsifying agents, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants. For example, a suitable vehicle may be water for injection, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to a formulation material(s) suitable for accomplishing or enhancing the delivery of the GRNF4 protein product as a pharmaceutical composition.

The primary solvent in a vehicle may be either aqueous or non-aqueous in nature. In addition, the vehicle may contain other formulation materials for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the vehicle may contain additional formulation materials for modifying or maintaining the rate of release of GRNF4 protein product, or for promoting the absorption or penetration of GRNF4 protein product across the blood-brain barrier.

Once the therapeutic pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration.

The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the intended route of administration, delivery format and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present GRNF4 protein products.

Effective administration forms, such as (1) slow-release formulations, (2) inhalant mists, or (3) orally active formulations are envisioned. The GRNF4 protein product pharmaceutical composition also may be formulated for parenteral administration. Such parenterally administered therapeutic compositions are typically in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising the GRNF4 protein product in a pharmaceutically acceptable vehicle. One preferred vehicle is physiological saline. The GRNF4 protein product pharmaceutical compositions also may include particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation.

A particularly suitable vehicle for parenteral injection is sterile distilled water in which the GRNF4 protein product is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation may involve the formulation of the GRNF4 protein

product with an agent, such as injectable microspheres, bio-erodible particles or beads, or liposomes, that provides for the controlled or sustained release of the protein product which may then be delivered as a depot injection. Other suitable means for the introduction of GRNF4 protein product include implantable drug delivery devices which contain the GRNF4 protein product.

The preparations of the present invention may include other components, for example parenterally acceptable preservatives, tonicity agents, cosolvents, wetting agents, complexing agents, buffering agents, antimicrobials, antioxidants and surfactants, as are well known in the art. For example, suitable tonicity enhancing agents include alkali metal halides (preferably sodium or potassium chloride), mannitol, sorbitol and the like. Suitable preservatives include, but are not limited to, benzalkonium chloride, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid and the like. Hydrogen peroxide may also be used as preservative. Suitable cosolvents are for example glycerin, propylene glycol and polyethylene glycol. Suitable complexing agents are for example caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin. Suitable surfactants or wetting agents include sorbitan esters, polysorbates such as polysorbate 80, tromethamine, lecithin, cholesterol, tyloxapal and the like. The buffers can be conventional buffers such as borate, citrate, phosphate, bicarbonate, or Tris-HCl.

The formulation components are present in concentration that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at slightly lower pH, typically within a pH range of from about 5 to about 8.

A pharmaceutical composition may be formulated for inhalation. For example, the GRNF4 protein product may be formulated as a dry powder for inhalation. GRNF4 protein product inhalation solutions may also be formulated in a liquefied propellant for aerosol delivery. In yet another formulation, solutions may be nebulized.

It is also contemplated that certain formulations containing GRNF4 protein product are to be administered orally. GRNF4 protein product which is administered in this fashion may be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule may be designed to release the active portion of the formulation at the point in the

gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional formulation materials may be included to facilitate absorption of GRNF4 protein product. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Another preparation may involve an effective quantity of GRNF4 protein product in a mixture with non-toxic excipients which are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or other appropriate vehicle, solutions can be prepared in unit dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional GRNF4 protein product formulations will be evident to those skilled in the art, including formulations involving GRNF4 protein product in combination with one or more other neurotrophic factors. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See, for example, Supersaxo et al. description of controlled release porous polymeric microparticles for the delivery of pharmaceutical compositions (International Publication No. WO 93/15722; International Application No. PCT/US93/00829) the disclosure of which is hereby incorporated by reference.

D. Administration of GRNF4

The GRNF4 protein product may be administered parenterally via a variety of routes, including subcutaneous, intramuscular, intravenous, transpulmonary, transdermal, intrathecal and intracerebral delivery. In addition, molecules that do not readily cross the blood-brain barrier may be given directly intracerebrally or otherwise in association with other elements that will transport them across the barrier. For example, the GRNF4 protein product may be administered intracerebroventricularly or into the brain or spinal cord subarachnoid space. GRNF4 protein product may also be administered intracerebrally directly into the brain parenchyma. GRNF4 protein product may be

administered extracerebrally in a form that has been modified chemically or packaged so that it passes the blood-brain barrier, or with one or more agents capable of promoting penetration of GRNF4 protein product across the barrier. For example, a conjugate of NGF and monoclonal anti-transferrin receptor antibodies has been shown to be transported to the brain via binding to transferrin receptors.

To achieve the desired level of GRNF4 protein product, repeated daily or less frequent injections may be administered, or GRNF4 protein product may be infused continuously or periodically from a constant- or programmable-flow implanted pump. Slow-releasing implants containing the neurotrophic factor embedded in a biodegradable polymer matrix can also deliver GRNF4 protein product. The frequency of dosing will depend on the pharmacokinetic parameters of the GRNF4 protein product as formulated, and the route and site of administration.

Regardless of the manner of administration, the specific dose may be calculated according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages may be ascertained through use of appropriate dose-response data.

The dosage regimen involved in a method for treating a specific injury or condition will be determined by the attending physician. Generally, an effective amount of the GRNF4 will be determined by considering various factors which modify the action of drugs, e.g., the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. See, Remington's Pharmaceutical Sciences, supra, at pages 697-773, herein incorporated by reference. For example, it is contemplated that if GFRα-3 is used to enhance GRNF4 action, then the GFRα-3 dose is selected to be similar to that required for GRNF4 therapy. If GFRα-3 is used to antagonize GRNF4 action, then the GFRα-3 dose would be several times the GRNF4 dose. Dosing may be one or more times daily, or less frequently, and may be in conjunction with other compositions as described herein. It should be noted that the present invention is not limited to the dosages recited herein.

It is envisioned that the continuous administration or sustained delivery of GRNF4 protein products may be advantageous for a given treatment. While continuous administration may be accomplished via a mechanical means, such as with an infusion pump, it is contemplated that other modes of continuous or near continuous administration may be practiced. For example, chemical derivatization or encapsulation may result in sustained release forms of the molecule which have the effect of continuous presence in the bloodstream, in predictable amounts, based on a determined dosage regimen. Thus, GRNF4 protein products include proteins derivatized or otherwise formulated to effectuate such continuous administration. Sustained release forms of GRNF4 will be formulated to provide the desired daily or weekly effective dosage.

It is further contemplated that a GRNF4 protein product may be administered in a combined form with GDNF and/or neurturin. Alternatively, the GRNF4 protein product may be administered separately from a neurotrophic factor, either sequentially or simultaneously.

As stated above, it is also contemplated that additional neurotrophic or neuron nurturing factors will be useful or necessary to treat some neuronal cell populations or some types of injury or disease. Other factors that may be used in conjunction with GRNF4 or a combination of GRNF4 and a neurotrophic factor such as GDNF, persephin or neurturin include, but are not limited to: mitogens such as insulin, insulin-like growth factors, epidermal growth factor, vasoactive growth factor, pituitary adenylate cyclase activating polypeptide, interferon and somatostatin; neurotrophic factors such as nerve growth factor, brain derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, neurotrophin-6, insulin-like growth factor, ciliary neurotrophic factor, acidic and basic fibroblast growth factors, fibroblast growth factor-5, transforming growth factor-ß, cocaine-amphetamine regulated transcript (CART); and other growth factors such as epidermal growth factor, leukemia inhibitory factor, interleukins, interferons, and colony stimulating factors; as well as molecules and materials which are the functional equivalents to these factors.

As described herein, GRNF4 may be used in the treatment of neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, and ALS, which result from the degeneration of specific neuronal populations. In addition, GRNF4 protein products

may be used in the treatment of nerve damage which may occur to one or more types of nerve cells by: (1) physical injury, which causes the degeneration of the axonal processes and/or nerve cell bodies near the site of injury; (2) temporary or permanent cessation of blood flow to parts of the nervous system, as in stroke; (3) intentional or accidental exposure to neurotoxins, for example, chemotherapeutic agents (e.g., cisplatinum) for the treatment of cancer or dideoxycytidine (ddC) for the treatment of AIDS; (4) chronic metabolic diseases, including diabetes or renal dysfunction. GRNF4 might also be used in the treatment of peripheral sensory neuropathy or neurological disorders associated with improperly functioning peripheral sympathetic nerves. It is also contemplated that GRNF4 may be used to treat diseases associated with bone loss such as osteoporosis, osteogenesis imperfecta or hypercalcemia of malignancy.

E. GRNF4 Cell Therapy and Gene Therapy

GRNF4 cell therapy, e.g., implantation of cells producing GRNF4, is also contemplated. This embodiment would involve implanting into patients cells capable of synthesizing and secreting a biologically active form of GRNF4. Such GRNF4-producing cells may be cells that are natural producers of GRNF4 or may be recombinant cells whose ability to produce GRNF4 has been augmented by transformation with a gene encoding the desired GRNF4 molecule. Such a modification may be accomplished by means of a vector suitable for delivering the gene as well as promoting its expression and secretion. In order to minimize a potential immunological reaction in patients being administered a GRNF4 protein or polypeptide of a foreign species, it is preferred that the natural cells producing GRNF4 be of human origin and produce human GRNF4. Likewise, it is preferred that the recombinant cells producing GRNF4 be transformed with an expression vector containing a gene encoding a human GRNF4 molecule.

Implanted cells may be encapsulated to avoid infiltration of surrounding tissue. Human or non-human animal cells may be implanted in patients in biocompatible, semipermeable polymeric enclosures or membranes that allow release of GRNF4, but that prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Alternatively, the patient's own cells, transformed to produce GRNF4 ex vivo, could be implanted directly into the patient without such

encapsulation.

Techniques for the encapsulation of living cells are familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. For example, Baetge et al. (International Publication No. WO 95/05452; International Application No. PCT/US94/09299 the disclosure of which is hereby incorporated by reference) describe membrane capsules containing genetically engineered cells for the effective delivery of biologically active molecules. The capsules are biocompatible and are easily retrievable. The capsules encapsulate cells transfected with recombinant DNA molecules comprising DNA sequences coding for biologically active molecules operatively linked to promoters that are not subject to down regulation in vivo upon implantation into a mammalian host. The devices provide for the delivery of the molecules from living cells to specific sites within a recipient. In addition, see U.S. Patent Numbers 4,892,538, 5,011,472, and 5,106,627, each of which is specifically incorporated herein by reference. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al., specifically incorporated herein by reference. See also, PCT Application WO 91/10470 of Aebischer et al., Winn et al., Exper. Neurol., 113:322-329, 1991, Aebischer et al., Exper. Neurol., 111:269-275, 1991; Tresco et al., ASAIO, 38:17-23, 1992, each of which is specifically incorporated herein by reference.

In vivo and in vitro gene therapy delivery of GRNF4 is also envisioned. In vivo gene therapy may be accomplished by introducing the gene encoding GRNF4 into cells via local injection of a polynucleotide molecule or other appropriate delivery vectors. (Hefti, J. Neurobiology,. 25:1418-1435, 1994). For example, a polynucleotide molecule encoding GRNF4 may be contained in an adeno-associated virus vector for delivery into the targeted cells (e.g., Johnson, International Publication No. WO 95/34670; International Application No. PCT/US95/07178 the disclosure of which is hereby incorporated by reference). The recombinant adeno-associated virus (AAV) genome contains AAV inverted terminal repeats flanking a DNA sequence encoding the neurotrophic factor operably linked to functional promoter and polyadenylation sequences.

Alternative viral vectors include, but are not limited to, retrovirus, adenovirus, herpes simplex virus and papilloma virus vectors. U.S. 5,672,344 (issued September 30,

1997, Kelley et al., University of Michigan), the disclosure of which is hereby incorporated by reference, describes an in vivo viral-mediated gene transfer system involving a recombinant neurotropic HSV-1 vector. U.S. 5,399,346 (issued March 21, 1995, Anderson et al., Department of Health and human Services), the disclosure of which is incorporated by reference herein, provides examples of a process for providing a patient with a therapeutic protein by the delivery of human cells which have been treated in vitro to insert a DNA segment encoding a therapeutic protein. Additional methods and materials for the practice of gene therapy techniques, the disclosures of which are incorporated by reference herein, are described in U.S. 5,631,236 (issued May 20, 1997, Woo et al., Baylor College of Medicine) involving adenoviral vectors; U.S. 5,672,510 (issued September 30, 1997, Eglitis et al., Genetic Therapy, Inc.) involving retroviral vectors; and U.S. 5,635,399 (issued June 3, 1997, Kriegler et al., Chiron Corporation) involving retroviral vectors expressing cytokines.

Nonviral delivery methods include liposome-mediated transfer, naked DNA delivery (direct injection), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation and microparticle bombardment (e.g., gene gun). Gene therapy materials and methods may also include inducible promoters, tissue-specific enhancer-promoters, DNA sequences designed for site-specific integration, DNA sequences capable of providing a selective advantage over the parent cell, labels to identify transformed cells, negative selection systems and expression control systems (safety measures), cell-specific binding agents (for cell targeting), cell-specific internalization factors, transcription factors to enhance expression by a vector as well as methods of vector manufacture. Such additional methods and materials for the practice of gene therapy techniques, the disclosures of which are incorporated by reference herein, are described in U.S. 4,970,154 (issued November 13, 1990, D.C. Chang, Baylor College of Medicine) electroporation techniques; WO 9640958 (published 961219 , Smith et al., Baylor College of Medicine) nuclear ligands; U.S. 5,679,559 (issued October 21, 1997, Kim et al., University of Utah Research Foundation) concerning a lipoprotein-containing system for gene delivery; U.S. 5,676,954 (issued October 14, 1997, K.L. Brigham, Vanderbilt University involving liposome carriers; U.S. 5,593,875 (issued January 14, 1997, Wurm et al., Genentech, Inc.) concerning methods for calcium phosphate

transfection; and U.S. 4,945,050 (issued July 31, 1990, Sanford et al., Cornell Research Foundation) wherein biologically active particles are propelled at cells at a speed whereby the particles penetrate the surface of the cells and become incorporated into the interior of the cells. Expression control techniques include chemical induced regulation (e.g., WO 9641865 and WO 9731899), the use of a progesterone antagonist in a modified steroid hormone receptor system (e.g., U.S. 5,364,791), ecdysone control systems (e.g., WO 9637609), and positive tetracycline-controllable transactivators (e.g., U.S. 5,589,362; U.S. 5,650,298; and U.S. 5,654,168).

It is also contemplated that GRNF4 gene therapy or cell therapy can further include the delivery of a second neurotrophic factor. For example, the host cell may be modified to express and release both GRNF4 and GDNF, or GRNF4 and neurturin. Alternatively, the GRNF4 and GDNF, or GRNF4 and neurturin, may be expressed in and released from separate cells. Such cells may be separately introduced into the patient or the cells may be contained in a single implantable device, such as the encapsulating membrane described above.

It should be noted that the GRNF4 formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges may be determined as described above.

EXAMPLES

Example 1

Construction of a cDNA Library

A cDNA library was constructed using the bones of osteoprotegerin (OPG)-deficient mice. Total RNA was isolated from the femurs and tibias of OPG knockout mice. The bones were dissected from six week old female mice and cleaned to remove muscle and connective tissue. The bones were homogenized with a Polytron homogenizer

in extraction buffer from Pharmacia's RNA Extraction Kit (Product No. 27-9270-01, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) with 7.2 µl ß-mercaptoethanol added to each 1 ml buffer used. After homogenization, insoluble materials were pelleted by a 5,000g spin at 15°C for 20 minutes. The supernatant was overlayed onto a 6 ml cushion of cesium trifluoroacetate solution (from the Pharmacia kit) in polyallomer tubes (Product No. 331372, Beckman Instruments, Inc., Fullerton, California, USA) and centrifuged at 30,000 rpm in a SW41Ti rotor at 15°C for 20 hours. RNA pellets were resuspended in diethyl pyrocarbonate treated water and ethanol precipitated. Poly A+ RNA was isolated from about 1 mg total RNA pooled from four female OPG knockout mice using Dynal's Dynabeads Oligo $(dT)_{25}$ (Product No. 610.05, Dynal, Oslo, Norway). The poly A+ RNA was purified by two rounds of binding to the Dynabeads.

OPG knockout crushed bone cDNA was synthesized from 3 µg of the polyA+ RNA using GIBCO/BRL's Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (Product No. 18248-013, GIBCO/BRL, Gaithersburg, MD, USA). The cDNA was oligo dT primed. The Not I primer adapter included in the kit was used to synthesize first strand cDNA.

Not I primer adapter:

5'- pGACTAGT TCTAGA TCGCGA GCGGCCGC CC $(T)_{15}$-3'

SpeI XbaI NruI NotI

Following second strand synthesis, a Sal I linker (Product No. 18248-013, GIBCO BRL) was added to the double stranded cDNA.

Sal I adapter:

5' - TCGACCCACGCGTCCG-3'

3' _____GGGTGCGCAGGCp-5'

SalI MluI

The cDNA with Sal I-Not I termini was size selected by column chromatography with the column included in the kit. The cDNA from column fraction 8 (also called pool 8) was ligated into pSport I (Product No. 18248-013, GIBCO BRL) cut with Sal I- Not I (included in the kit). Ligated DNA was electroporated into Electromax DH10B cells (Product No.18290-015, GIBCO BRL). The average insert size was 2.3kb.

Example 2

Identification and Isolation of murine GRNF4

Following rearray of the OPG knockout crushed bone library and subtraction with common cDNA probes, several thousand clones from the library were sequenced from the 5' end. For subtraction, cDNA from some of the library's most abundant clones was used to make radioactive probes for hybridization to filters containing an array of 34.000 library clones. Select clones that did not hybridize to these probes were sequenced. One clone, smcb2-00011-d2 (Figure 1a), encoded an open reading frame (ORF) which showed homology to the C-terminal active domain of GDNF. All seven cysteine residues characteristic of TGF-β family members were present in the predicted open reading frame encoded by smcb2-00011-d2. The predicted ORF was 47% identical to neurturin, which is structurally related to GDNF (Figure 1b). An RXXR (Arg-Xaa-Xaa-Arg) cleavage site (commonly found in TGF-β family members) was also present, and thus, the expressed sequence tag (EST) was predicted to encode the sequence of the active portion of a novel GDNF family member. After confirmation of the double stranded sequence, the molecule was designated GRNF4 (for GDNF-related neurotrophic factor 4).

The 3' untranslated region of GRNF4 was obtained by further sequencing the smcb2-00011-d2 clone (Figure 1c). The 5' end of GRNF4 was cloned by 5' Rapid Amplification of cDNA Ends (i.e., RACE, as described in Frohman, M.A. (1993) Methods of Enzymology 218:340-358) from the smcb2-00011-d2 clone. Four oligonucleotides were synthesized based on the cDNA sequence of the smcb2-00011-d2 clone. These oligonucleotides were used to screen potential sources for 5'RACE to obtain the full length coding region of GRNF4.

Oligonucleotide Probe	Sequence
2037-99	5-TCC GAC GAG CTG ATA CGT TTC C-3
2038-01	5-GGA GCT GTT CCA GGT AGG GCA A-3
2038-02	5-AGC ACG CTC CCA GCA CGA TCT C-3
2038-03	5-TGG GAC TGT TGG TCA GTG GTT C-3

RACE Oligos	Sequence
AP1*	5-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3
AP2*	5-ACT CAC TAT AGG GCT CGA GCG GC-3

*Oligonucleotides obtained from Clontech

Oligonucleotide pair (2037-99 + 2038-01) was used to survey potential sources, amplifying a fragment of 306bp. Marathon-Ready cDNA from Mouse Brain (Clontech Product No. 7450-1) showed positive identification of the fragment , indicating it was an appropriate source for 5' RACE. PCR materials were as follows:

5 µl Marathon-Ready Mouse Brain cDNA(0.1µg/µl)

1 µl oligonucleotide 2037-99 (20µM)

1 µl oligonucleotide 2038-01 (20µM)

1 µl dNTP (10mM), (Clontech, Product No. 7411-1)

5 µl 10x cDNA PCR Reaction Buffer, (Clontech, Product No. 7411-1)

1 µl Advantage cDNA Polymerase Mix(50x), (Clontech, Product No. 7411-1)

36 µl dH_2O

The *conditions for PCR amplification were denaturation at 94°C for two minutes, followed by 30 cycles of 94°C for 30 seconds (denature), to 62°C for 30 seconds (anneal), to 72°C for 30 seconds (extend). Finally, the reaction was incubated at 72°C for 7 minutes for a final extension. (*94°C/2min; [94°C/30sec, 62°C/30sec, 72°C/2min] x 30 cycles; 72°C/7min.)

Initial 5' RACE was performed with oligos 2038-03 + AP1. The conditions for PCR amplification were as described above. The PCR product from the 5' RACE was purified (e.g., Qiaquick PCR Purification Kit, Qiagen, Hilden, Germany, Product No. 28104). The purified product was diluted to 0.1 μg/μl.

The initial RACE product was nested with oligos 2038-01 + AP2 using the same reaction conditions described above (+ or -) 5μl 0.5M GC Melt (Clontech, Product No. 8419-1) with adjusted water volume. Following gel electrophoresis of the nested PCR products, bands at approximately 900bp and 1500bp appeared with both + or - GC melt. The bands were extracted, and the DNA was purified with the Qiaquick Gel Extraction Purification Kit (Qiagen, Product No. 28704).

Following verification of the fragments with internal check PCR, the fragments were subcloned into a PCR cloning plasmid (i.e., pCR2.1 TOPO-TA Cloning Kit, Invitrogen, Product No. K4500-40) following manufacturer instructions. Following transformation of bacteria, clones were purified with the Spin Miniprep Kit (Qiagen, Product No. 27104) following manufacturer instructions. Clones were subjected to an internal check PCR using oligonucleotide pair (2037-99 + 2038-01) with PCR conditions noted above, and EcoRI Digest (0.5ug DNA, 2μl 10xBuffer H, 1μl EcoRI (10u/μl, Boehringer Mannheim, Indianapolis, IN. USA, Product No. 703 737), dH_2O to 20μl.) DNA was digested by incubating at 37°C for 30 minutes. Digested DNA was analyzed on 1%Agarose/1xTBE (GIBCO BRL, Product No. 15510-027).

Clones were submitted for sequencing. Several clones were obtained which extended the 5' end of the original EST. The sequence for the novel gene is depicted in Figure 2. The starting Met (predicted ORF) starts at bp position 217. The predicted 224 amino acid ORF encoded by this full length murine GRNF4 gene contained a predicted signal peptide with an upstream stop. Within the GDNF family, full length GRNF4 was most highly related to neurturin, being approximately 38 % identical at the amino acid level. A comparison of murine GRNF4 and neurturin amino acid sequences is depicted in Figure 4. A comparison of murine GRNF4 amino acid sequence to those of neurturin, persephin and GDNF is depicted in Figure 5. The relative sizes of the proteins are as follows:

GDNF murine:	241aa
Neurturin murine:	196aa
Persephin murine:	157aa
GRNF4 murine:	224aa

Example 3

Identification and Isolation of human GRNF4

The putative coding region of mouse clone, smcb2-00011-d2 (Example 2, Figure 1A), was used to search public databases for homologous sequences. A homologous sequence is defined as a sequence with sufficient identity (>20-25% at the amino acid level) to suggest that the sequences are related, (i.e. the genes encoding the sequences are derived from duplication of a common ancestor gene). Two human genomic clones (Accession Nos. AC005038 and AC005051) contained regions that are highly homologous to smcb2-00011-d2 (i.e., 83% or greater identity (BLASTP) at amino acid level, representing the nucleic acid homology between human and mouse GRNF4 over the region spanning the original EST). This region included the active portion of mouse GRNF4 as well as a 3' translation stop codon. Furthermore, about 400 bp upstream of this region was a region homologous to the 5' end of the mouse cDNA, indicating the presence of an intron. The translation start codon was found in this upstream region. Thus, regions surrounding the translation start and stop codons were identified based on homology to mouse cDNA sequence.

Several oligonucleotide primers surrounding the translation start and stop codons were designed to clone the human cDNA coding region by PCR method. The probes are presented in Table 2.

Table 2

Oligonucleotide Primers for Amplification of Human GRNF4 Gene

Oligonucleotide Probe	Sequence
2058-59	5-GGT GGG GGA ACA GCT CAA CAA T-3
2058-60	5-CAA CAA TGG CTG ATG GGC G-3
2020-27	5-GTA AGG GTC CAG TCT GCA AAG-3
2035-28	5-TCA GCC CAG GCA GCC GCA G-3

Because GRNF4 was found to be highly expressed in human placenta (see Example 8 below, Figure 9), the marathon-ready cDNA (Clontech, Product No. 7411-1) from human placenta was used as a template source. Oligonucleotide pair (2058-59 + 2020-27) was used to perform the initial PCR reaction. The materials and conditions were as follows:

5 µl Marathon-Ready Human placenta cDNA(0.1µg/µl) (Clontech, Product No. 7411-1)

5 µl GC-melt (Clontech, Product No. 8419-1)

1 µl oligonucleotide 2058-59 (20µM)

1 µl oligonucleotide 2020-27 (20µM)

1 µl dNTP(10mM), (Clontech, Product No. 7411-1)

5 µl 10x cDNA PCR Reaction Buffer, (Clontech, Product No. 7411-1).

1 µl Advantage cDNA Polymerase Mix (50x), (Clontech, Product No. 7411-1)

31µl dH_2O

94°C/2min; [94°C/30sec,58°C/30sec,72°C/1 min.]x25 cycles; 72°C/7 min

This PCR reaction produced no discernible bands. Thus, a 1:10 dilution of this PCR reaction was used as a template to perform a secondary PCR reaction with nested primer pair (2058-60 + 2035-28). The materials and conditions were as follows:

1 µl 1:10 dilution of initial PCR reaction

5 µl GC-melt (Clontech, Product No. 8419-1)

1 µl oligonucleotide 2058-60 (20µM)

1 µl oligonucleotide 2035-28 (20µM)

1 µl dNTP(10mM), (Clontech, Product No. 7411-1)

5 µl 10x cDNA PCR Reaction Buffer, (Clontech, Product No. 7411-1)

1 µl Advantage cDNA Polymerase Mix(50x), (Clontech, Product No. 7411-1)

35µl dH_2O

94°C/2min; [94°C/30sec,58°C/30sec,72°C/45sec.]x25 cycles; 72°C/7 min

This PCR reaction produced a nucleotide having about 730 base pairs. This fragment was gel purified using the Qiaquick gel extraction Kit (Qiagen) and cloned into the pCR2.1 TOPO-TA Cloning Kit (Invitrogen).

The nucleotide sequence for human GRNF4 is depicted in Figure 6. The amino acid sequence for human GRNF4 is depicted in Figure 7.

Figure 8 depicts a protein sequence comparison between mouse and human GRNF4. The comparison demonstrates that the mouse and human sequences are 78.7% similar and 77.4% identical over the full length coding region.

Example 4

Production of murine GRNF4

The DNA fragment encoding the mature form of GRNF4 was amplified using PCR. The oligonucleotides used as primers for this reaction were designed such that XbaI and XhoI restriction sites were placed at the 5' and 3' ends of the gene respectively. The amplified PCR product was digested with the appropriate enzymes and cloned into a plasmid or expression vector as described above under recombinant expression of GRNF4.

The GRNF4 plasmid was then transformed into an E. coli host cell for expression of the GRNF4 protein. Following induction, expression of the GRNF4 protein can be visualized by SDS PAGE.

GRNF4 was expressed in inclusion bodies in E. coli. Inclusion bodies were solubilized in 6M guanidine HCl, 50 mM Tris, 8 mM DTT for one hour at room

temperature. The solubilized inclusion bodies were diluted 25 fold into 2 M urea, 50 mM Tris, 160 mM arginine, 3 mM cysteine, pH 8.5 and stirred overnight in the cold (4°C). The mixture was clarified by centrifugation, concentrated about 10 fold, and diluted 3 fold with 1.5 M urea, 5 mM Tris, pH 9. The resulting mixture was clarified by centrifugation, pH adjusted to 6.8 (with phosphoric acid) and loaded onto an ion exchange column (SP-Sepharose column, Amersham Pharmacia Biotech) equilibrated in 10 mM Na phosphate, 0.2 M arginine, pH 6.8. After loading and washing the column with the same buffer, the GRNF4 was eluted off the column using a gradient from 0 to 1 M NaCl in the same buffer. Peak fractions were pooled and pH adjusted to 4.5. Ammonium sulfate was added to 0.8 M, and the mixture was loaded onto a hydrophobic interaction chromatography column (Butyl Toyopearl chromatography column, TosoHaas, Montgomeryville, PA, USA) equilibrated in 10 mM Na acetate, 0.8 M ammonium sulfate. After loading and washing with the same buffer, the GRNF4 was eluted using a gradient from 0.8 M to 0 M ammonium sulfate in the same buffer. The GRNF4 was then dialyzed into 10 mM Na acetate, 150 mM NaCl, pH 4.5.

Example 5

Preparation and Iodination of GRNF4

The predicted mature form GRNF4 was expressed in E. Coli as described in Example 4. This molecule was radio-labeled with [^{125}I] using lactoperoxidase reagents. Figure 11 shows the autoradiograph of the [^{125}I]-labeled GRNF4 fractionated by a 16% SDS-PAGE under non-reducing (NR) and reducing conditions. The apparent molecular weight of the [^{125}I]GRNF4 is ~24 kD under non-reducing condition (homodimer) and ~12 kD under reducing condition (monomer).

Example 6

GRNF4 Binding to Cells Expressing GFRα-3

A binding assay was performed in accordance with an assay method previously described by Jing et al. (Journal Of Cell Biology, 110, 283-294, 1990). The assay

involved the binding of [^{125}I]GRNF4 (as described in Example 5) to NSR-1, NSR-5, and NSR-19 cells. These cells are clones of Neuro-2a cells (ATCC Number CCL 131) which had been transfected to express GFRα-3. To generate these clones, Neuro-2a cells were transfected with a GFRα-3 expression vector (GFRα-3 cDNA cloned in pBKRSV, Stratagene, La Jolla, CA). using the Calcium Phosphate Transfection System (GIBCO/BRL) according to the manufacturer's directions. Transfected cells were selected for expression of the plasmid by growing in 400 mg/ml G418 antibiotic (Sigma). G418 resistant clones were expanded and analyzed for expression of GFRα-3 by Northern blot by using the GFRα-3 cDNA as probe.

The cloned cells expressing GFRα-3 (i.e., NSR-1, NSR-5, and NSR-19) were seeded 24 hours before the assay in 24-well Costar tissue culture plates which were pre-coated with polyornithine and laminin at a density of 1.5 x 10^5 cells/cm^2. Cells were left on ice for 5 to 10 minutes, washed once with ice-cold washing buffer (Dolbeco Modified Eagle's Medium (GIBCO/BRL) containing 25 mM HEPES, pH 7.4) and incubated with 0.2 mL of binding buffer (washing buffer supplied with 2mg/ml BSA) containing 0.1 nM of [^{125}I]GRNF4 in the presence or absence of 50 nM of unlabeled GRNF4 at 4°C for four hours. Cells were washed four times with 0.5 mL ice-cold washing buffer and lysed with 0.5 mL of 1 M NaOH. The lysates were counted in a 1470 Wizard Automatic Gamma Counter.

The results of this analysis are presented in Figure 12. The experiment demonstrated that recombinant GRNF4 specifically binds to cells expressing GFRα-3. In addition, cells expressing higher levels of GFRα-3 (e.g., NSR-5) bind GRNF4 more efficiently.

Example 7

GRNF4 Binding to BiaCore Surface Coated by Soluble GFRα-3 Protein

GRNF4 was found to specifically bind a surface coated by a soluble flag-tagged GFRα-3 receptor (a surface plasmon resonance analysis, BIACORE® biosensor-based analytical instrument for studying interactions, BiaCore AB, Uppsala, Sweden). GRNF4

did not specifically bind GFRα-1 or GFRα-2 receptor proteins. The results of this analysis are presented in Figure 13.

Example 8

Tissue Distribution of GRNF4 mRNA

Tissue distributions of GRNF4 in both mouse and human were studied using Northern blot analysis. The human GRNF4 probe was prepared by a PCR reaction from human genomic DNA (Clontech). The probe region corresponds to nucleotides 484 to 672 in Figure 6. This fragment was generated using appropriate primers, cloned into pCR2.1 (Clontech), and sequence verified. 20 ng of EcoRI fragment from this clone was labeled with ^{32}P-dCTP using Rediprime II kit (Amersham). Human Multiple Tissue Northern Blot (Figure 9A, Clontech) and Human Multiple Tissue Northern Blot II (Figure 9B, Clontech) were hybridized in 10 ml of Expresshyb solution with $4X10^6$ cpm/ml of probe at 60°C for 14 hours. The blots were washed twice in 0.5%SDS, 2XSSC solution for 30 minutes at room temperature. They were further washed three times in 0.1%SDS, 0.1XSSC for 30 minutes at 55°C. These membranes then were exposed under an X-Omat AR film (Kodak) for three days at -80°C. The probe detected two strong bands at 4.3 and 1.7 kB especially in placenta, pancreas, and prostate (Figure 9). Somewhat weaker expression was also observed in testis, ovary, small intestine, colon (mucosal line), kidney, and heart. Interestingly, only the 1.7 kB band was observed in kidney. More tissues were surveyed with Human RNA master blot (Clontech, data not shown), and the results confirmed the Northern blot analysis. Furthermore, additional expression signals were observed in pituitary gland, fetal kidney, fetal lung, and adult trachea.

A portion of mouse cDNA (from nucleotide 649 to 954 in Figure 2) was labeled with ^{32}P-dCTP in a PCR labeling reaction. The same hybridization and wash protocol was used as in human Northern blot analysis, except $4X10^6$ cpm/ml of probe was used to hybridize Mouse multiple tissue northern blot (Clontech, Figure 10). 1.4 and 1.0 bands were detected in testis. As in human expression analysis, Mouse RNA master blot (Clontech) was used to survey more tissues. Testis and uterus showed strong GRNF4 expression, whereas thyroid, prostate, and epididymus showed moderate expression.

Example 9

Chemical Crosslinking of GRNF4 with GFRα-3

In order to study the binding properties and molecular characteristics of GRNF4, chemical crosslinking experiments were performed. The experiments involved [^{125}I]GRNF4 linked to a soluble GFRα-3/human Fc fusion protein or linked to GFRα-3 and Ret receptors expressed on the surface of NSR-5 cells (described in Example 6).

For crosslinking using the soluble GFRα-3 receptors, either [^{125}I]GDNF or [^{125}I]GRNF4 was added to 1 ml of 1x conditioned media of 293T cells which express the GFRα-1, GFRα-2, or GFRα-3/hFc fusion proteins, to a final concentration of 2 nM. The soluble GFRα-3/hFc protein was transiently expressed using 293T cells (293 cells, ATCC CRL-1573 expressing the SV40 large T antigen; modified by and obtained from Cell and Molecular Technologies, Inc., Lavallette, New Jersey, USA) by transfection of the plasmids containing the fused GFRα-human Fc cDNAs. Transfection of 293T cells was performed using the Calcium Phosphate Transfection System (GIBCO/BRL, Gaithersburg, MD) according to the manufacturers instructions. The cells were incubated in the presence or absence of 1 μM of unlabeled GDNF, neurturin, or GRNF4 at 4°C for four hours. A chemical crosslinker, Bis suberate (BS^3 Pierce, Rockford, IL), was added to a final concentration of 1 mM and further incubated at room temperature for 30 minutes. The crosslinking reaction was quenched by incubating with 50 mM of glysine at room temperature for 15 more minutes. The crosslinked [^{125}I]GRNF4-GFRα-3/hFc complex was precipitated by Protein A sepharose beads and fractionated on a 7.5% SDS-PAGE (bis:acrylamide = 1:200). The results are depicted in Figure 14.

To evaluate the binding of [^{125}I]GRNF4 to GFRα-3 and Ret receptors expressed on the surface of cells, the following cells were used: NGR-38, NNR-9, and NSR-5 cells. These clones were generated as described in Example 6, above. Neuro-2a cells were transfected with expressing plasmids containing each of the GFRα cDNAs using the Calcium Phosphate Transfection System (GIBCO/BRL) according to the manufacturer's

directions. Transfected cells were selected for expression of the plasmid by growing in 400 mg/ml G418 antibiotic (Sigma). G418 resistant clones were expanded and analyzed for expression of each of the GFRαs by Northern blot by using each individual GFRα cDNA as probe. NGR-38 cells express GFRα and Ret, NNR-9 cells express GFRα-2 and Ret and NSR-5 cells express GFRα-3 and Ret.

The cells were seeded 24 hours prior to the experiment in 6-well tissue culture plates at a density of 1.5 x 10^5 cells/cm^2. Cells were left on ice for 10-15 minutes, washed once with washing buffer (described above), and incubated with 2 nM of [^{125}I]GRNF4 in the presence or absence of 1 μM of unlabeled GRNF4 at 4°C for four hours. BS^3 was added to 1 mM and incubated at 4°C for 30 minutes. The crosslinking reaction was quenched by incubating with 50 mM of glysine at room temperature for 15 more minutes. The cells were washed four times with ice-cold washing buffer and lysed with Triton X-100 lysis buffer. The cell lysates were immunoprecipitated using an anti-Ret antibody and the immunoprecipitates were resolved on a 7.5% SDS-PAGE.

The results of the cell binding analysis are shown in Figure 15. GFRα-1/hFc and GFRα-2/hFc fusion proteins did not exhibit any GRNF4 binding capacity. GFRα-3 protein bound strongly to [^{125}I]GRNF4. GFRα-1 and GFRα-2 expressed in NGR-38 and NNR-9 cells did not bind GRNF4. GFRα-3 receptors expressed on the surface of NSR-5 cells bound GRNF4 efficiently. In addition, in the presence, but not the absence of GFRα-3, GRNF4 also binds the Ret receptor. Crosslinked [^{125}I]GRNF4-GFRα-3 complex was co-precipitated with the [^{125}I]GRNF4-Ret complex using an anti-Ret antibody, suggesting strong non-covalent interactions between GFRα-3 and Ret in the presence of GRNF4. This binding was almost completely inhibited by 1 μM of unlabeled GRNF4, indicating a specific binding of native GRNF4 to the expressed receptors.

Example 10

Autophosphorylation of Ret Receptor Protein Tyrosine Kinase Induced by GRNF4

GRNF4-induced Ret tyrosine phosphorylation in NSR-5 cells was determined by immunoblotting. NGR-38, NNR-9, or NSR-5 cell cultures (as described in Example 9)

were treated with 50 nM of GDNF, neurturin, persephin, and GRNF4 at 37°C for ten minutes. Cells were lysed, and the cell lysates were subjected to immunoprecipitation using an anti-Ret antibody and Western blot using an anti-phosphotyrosine antibody. The results of this analysis are shown in Figure 16. As in NGR-38 cells treated with GDNF and NNR-9 cells treated with neurturin, treatment of NSR-5 cells which express GFRα-3 with GRNF4 efficiently induced tyrosine phosphorylation on the Ret receptor protein tyrosine kinases.

Example 11

Dose Dependence and Kinetics of GRNF4-Induced Ret Phosphorylation

NSR-5 cells were treated with various concentrations of GRNF4 at 37°C for 10 minutes (Figure 17 panel A), or with 2 nM of GRNF4 at 37°C for various periods of time as indicated in the figure (Figure 17 panel B). Cells were lysed, and the cell lysates were subjected to immunoprecipitation using an anti-Ret antibody and Western blot analysis using an anti-phosphotyrosine antibody. The intensity of tyrosine phosphorylation of the Ret receptors induced by GRNF4 varies depending on the concentration of the GRNF4 used and the time periods of the treatment. GRNF4 can elicit Ret phosphorylation in NSR-5 cells with a concentration of GRNF4 as low as 2 pM, indicating a high affinity interaction between GRNF4 and its receptors. In addition, GRNF4 is apparently able to elicit Ret phosphorylation within a minute, demonstrating a very quick activation of the Ret kinase induced by GRNF4.

Example 12

Stabilization of GRNF4 with GFRα-3

GFRα receptors are anchored to the plasmic membrane by glycosylphosphotidyl-inositol (GPI) linkages. Although it is not clear whether or not soluble GFRαs exist *in vivo*, some GPI-linked molecules are partially released from the membrane. The fact that GRNF4 binds to and can be cross-linked with soluble GFRα-3 receptors indicates that

formation of the GRNF4-GFRα-3 complexes may help to stabilize either or both of the molecules. Formation of the GRNF4- GFRα-3 complexes may also help to maintain a constant concentration of GRNF4 *in vivo*, this reduces any potential toxicity associated with a high concentration of GRNF4.

Example 13

GRNF4 Percent Identity

A Washington University Blast search was performed using the murine GRNF4 amino acid sequence. The search provided the results presented in Table 3.

Table 3
BLASTP Search Results

```
BLASTP 2.0a13MP-WashU [10-Jun-1997] [Build 23:08:12 Jun 10 1997]

Reference: Gish, Warren (1994-1997). unpublished.
Altschul, et al. (1990). Basic local alignment search tool. J. Mol.
Biol. 215:403-10.

Query= /tmp/seq15527.seq (225 letters)

Database: Nonredundant Protein 307,932 sequences; 97,950,973 total
letters.
```

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
SW:NRTN_HUMAN	Q99748 homo sapiens (human). neurturin pr...	243	1.1e-20	1
SW:NRTN_MOUSE	P97463 mus musculus (mouse). neurturin pr...	243	1.1e-20	1
GP:AF040960_1	AF040960 Mus musculus persephin mRNA, com...	236	6.0e-20	1
GP:AF040962_1	AF040962 Homo sapiens persephin mRNA, com...	231	2.0e-19	1
GP:AF040961_1	AF040961 Rattus norvegicus persephin mRNA...	224	1.1e-18	1
SW:GDNF_MOUSE	P48540 mus musculus (mouse). glial cell l...	172	3.7e-13	1
GP:D88264_1	D88264 Mus musculus mRNA for neurotrophic...	172	3.7e-13	1
PIR:I67605	glial cell line-derived neurotrophic fact...	168	9.7e-13	1
PIR:I53427	glial cell line-derived neurotrophic fact...	168	9.7e-13	1
SW:GDNF_RAT	Q07731 rattus norvegicus (rat). glial cel...	167	1.2e-12	1
SW:GDNF_HUMAN	P39905 homo sapiens (human). glial cell l...	162	4.2e-12	1
PIR:PQ0452	extensin-like protein - Persian tobacco (...	89	0.00071	1
GP:A31038_1	A31038 N.alata mRNA for PRP3 (proline-ric...	89	0.00071	1
GP:HUMACHEA_1	M76539 Human acetylcholinesterase (ACHE) ...	87	0.0012	1
GP:D85682_1	D85682 Bos taurus mRNA for synaptojanin, ...	112	0.0019	1

```
Descriptions of 162 database sequences were not reported due to the
limiting value of parameter V = 5.

SW:NRTN HUMAN Q99748 homo sapiens (human). neurturin precursor. 7/98
          Length = 197
Score = 243 (85.5 bits), Expect = 1.1e-20, P = 1.1e-20
```

```
Identities = 56/113 (49%), Positives = 68/113 (60%)

SW:NRTN MOUSE P97463 mus musculus (mouse). neurturin precursor. 11/97
          Length = 195
Score = 243 (85.5 bits), Expect = 1.1e-20, P = 1.1e-20
Identities = 81/206 (39%), Positives = 102/206 (49%)

GP:AF040960 1 AF040960 Mus musculus persephin mRNA, complete cds;
          neurotrophic factor; PSP. Length = 156
Score = 236 (83.1 bits), Expect = 6.0e-20, P = 6.0e-20
Identities = 53/123 (43%), Positives = 69/123 (56%)

GP:AF040962 1 AF040962 Homo sapiens persephin mRNA, complete cds;
          neurotrophic factor; PSP. Length = 156
Score = 231 (81.3 bits), Expect = 2.0e-19, P = 2.0e-19
Identities = 54/112 (48%), Positives = 70/112 (62%)

GP:AF040961 1 AF040961 Rattus norvegicus persephin mRNA, complete cds;
          neurotrophic factor; PSP. Length = 156
Score = 224 (78.9 bits), Expect = 1.1e-18, P = 1.1e-18
Identities = 47/98 (47%), Positives = 63/98 (64%)

SW:GDNF MOUSE P48540 mus musculus (mouse). glial cell line-derived
          neurotrophic factor precursor. 11/97 Length = 211
Score = 172 (60.5 bits), Expect = 3.7e-13, P = 3.7e-13
Identities = 47/147 (31%), Positives = 72/147 (48%)
```

The BLAST search was performed using the default parameters of the Genetic Computer Group (see, GCG, University of Wisconsin, Madison, WI). In this search, the query sequence (the GRNF4 224 amino acid sequence) was compared to 307,932 sequences in the Nonredundant Protein database. The highest homology matches were to human and mouse neurturin; mouse, human, and rat persephin; and mouse human and rat glial-derived neurotrophic factor. These three molecules make up the previously known members of the GDNF family of neurotrophic factors. These results suggest that GRNF4 is a novel member of the GDNF family, representing the fourth member of this family.

As seen from this data, a "smallest sum probability" value for significant matches indicating a related family member are from 4.2e-12 to 1e-20. The smallest sum probability for a protein equivalent to mouse or human GRNF4 would have a score of 4.9e-21 to 1.2e-241 (the later value obtained for a match of a human molecule, e.g., neurturin, to itself).

Example 14

GRNF4 Consensus Sequence

A GRNF4 consensus sequence is depicted in Figure 18. This sequence is based upon the comparison of the amino acid sequences of mouse and human GRNF4, wherein "Xaa" represents a deletion, addition or substitution of an amino acid residue.

While the present invention has been described in terms of preferred embodiments and exemplary polynucleotide molecules and amino acid sequences, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims encompass all such equivalent variations which come within the scope of the invention as claimed. The disclosures of the references cited in this document are incorporated by reference herein

CLAIMS

What is claimed is:

1. An isolated and purified protein product comprising an amino acid sequence selected from the group consisting of:

a) an amino acid sequence of Figure 3 (SEQ ID NO:__),
b) amino acid residues 81 through 224 of Figure 3 (SEQ ID NO:__),
c) amino acid residues 109 through 224 of Figure 3 (SEQ ID NO:__),
d) amino acid residues 112 through 224 of Figure 3 (SEQ ID NO:__),
e) amino acid residues 119 through 224 of Figure 3 (SEQ ID NO:__),
f) amino acid residues 129 through 224 of Figure 3 (SEQ ID NO:__),
g) an amino acid sequence of Figure 7 (SEQ ID NO:__),
h) amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),
i) amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),
j) amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),
k) amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),
l) amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),
m) an amino acid sequence of Figure 18 (SEQ ID NO:__),
n) amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),
o) amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),
p) amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), and
q) amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__),

and wherein the protein product binds GDNF family receptor-alpha-3 (GFRα-3).

2. A protein product of Claim 1 which is glycosylated.

3. A protein product of Claim 1 which is non-glycosylated.

4. A pharmaceutical composition comprising a protein product of claim 1, 2 or 3 in combination with a pharmaceutically acceptable carrier.

5. An isolated polynucleotide molecule which encodes a protein product that is at least 82% identical in amino acid sequence to a protein product of claim 1, wherein said protein product binds GDNF family receptor-alpha-3 (GFRα-3), and wherein said percent identity is determined by GAP, BLAST or FASTA using standard default parameters.

6. An isolated polynucleotide molecule which encodes a protein product that is at least 82% identical in amino acid sequence to a protein product of claim 1, wherein said protein product binds GDNF family receptor-alpha-3 (GFRα-3), and wherein said percent identity is determined by BLASTP using standard default parameters.

7. An isolated polynucleotide molecule selected from the group consisting of:

a) a molecule comprising the nucleotides of Figure 2 (SEQ ID NO:__) or its complement,

b) a molecule encoding a polypeptide comprising amino acid residues 81 through 224 of Figure 3 (SEQ ID NO:__),

c) a molecule encoding a polypeptide comprising amino acid residues 109 through 224 of Figure 3 (SEQ ID NO:__),

d) a molecule encoding a polypeptide comprising amino acid residues 112 through 224 of Figure 3 (SEQ ID NO:__),

e) a molecule encoding a polypeptide comprising amino acid residues 119 through 224 of Figure 3 (SEQ ID NO:__),

f) a molecule encoding a polypeptide comprising amino acid residues 129 through 224 of Figure 3 (SEQ ID NO:__),

g) a molecule comprising the nucleotides of Figure 6 (SEQ ID NO:__) or its complement,

h) a molecule encoding a polypeptide comprising amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),

i) a molecule encoding a polypeptide comprising amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),

j) a molecule encoding a polypeptide comprising amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),

k) a molecule encoding a polypeptide comprising amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),

l) a molecule encoding a polypeptide comprising amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),

m) a molecule encoding a protein product comprising an amino acid sequence of Figure 3 (SEQ ID NO:__),

n) a molecule encoding a protein product comprising an amino acid sequence of Figure 7 (SEQ ID NO:__), and

o) a molecule encoding a protein product comprising an amino acid sequence of Figure 18 (SEQ ID NO:__)

p) a molecule encoding a protein product comprising an amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),

q) a molecule encoding a protein product comprising an amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),

r) a molecule encoding a protein product comprising an amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), and

s) a molecule encoding a protein product comprising an amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__).

8. An isolated polynucleotide molecule selected from the group consisting of:

a) a molecule which hybridizes under stringent conditions to a complementary sequence of a molecule of Claim 5; and

b) a molecule which but for the degeneracy of the genetic code would hybridize under stringent conditions to a complementary sequence of a molecule of Claim 5,

and wherein said isolated polynucleotide molecule encodes a protein product that binds GDNF family receptor-alpha-3 (GFRα-3).

9. A vector comprising a polynucleotide molecule according to claim 5. 6. 7 or 8.

10. A vector according to claim 9, further comprising one or more operational elements capable of effecting the amplification or expression of said polynucleotide molecule.

11. A vector comprising polynucleotide molecule selected from the group consisting of:

a) a molecule encoding a polypeptide comprising amino acid residues 81 through 224 of Figure 3 (SEQ ID NO:__),

b) a molecule encoding a polypeptide comprising amino acid residues 109 through 224 of Figure 3 (SEQ ID NO:__),

c) a molecule encoding a polypeptide comprising amino acid residues 112 through 224 of Figure 3 (SEQ ID NO:__),

d) a molecule encoding a polypeptide comprising amino acid residues 119 through 224 of Figure 3 (SEQ ID NO:__),

e) a molecule encoding a polypeptide comprising amino acid residues 129 through 224 of Figure 3 (SEQ ID NO:__),

f) a molecule encoding a polypeptide comprising amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),

g) a molecule encoding a polypeptide comprising amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),

h) a molecule encoding a polypeptide comprising amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),

i) a molecule encoding a polypeptide comprising amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),

j) a molecule encoding a polypeptide comprising amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),

k) a molecule encoding a protein product comprising an amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),

l) a molecule encoding a protein product comprising an amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),

m) a molecule encoding a protein product comprising an amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), and

n) a molecule encoding a protein product comprising an amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__).

12. A genetically engineered host cell comprising a polynucleotide molecule according to claim 5. 6. 7 or 8.

13. A genetically engineered host cell which expresses a protein product comprising an amino acid sequence selected from the group consisting of:

a) an amino acid sequence of Figure 3 (SEQ ID NO:__),

b) amino acid residues 81 through 224 of Figure 3 (SEQ ID NO:__),

c) amino acid residues 109 through 224 of Figure 3 (SEQ ID NO:__),

d) amino acid residues 112 through 224 of Figure 3 (SEQ ID NO:__),

e) amino acid residues 119 through 224 of Figure 3 (SEQ ID NO:__),

f) amino acid residues 129 through 224 of Figure 3 (SEQ ID NO:__),

g) an amino acid sequence of Figure 7 (SEQ ID NO:__),

h) amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),

i) amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),

j) amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),

k) amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),

l) amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),

m) an amino acid sequence of Figure 18 (SEQ ID NO:__),

n) amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),

o) amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),

p) amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), and

q) amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__),

and wherein the protein product binds GDNF family receptor-alpha-3 (GFRα-3).

14. A genetically engineered host cell comprising a vector of claim 10.

15. A genetically engineered host cell of Claim 12 wherein said cell is suitable for human implantation and wherein said cell expresses and secretes said polynucleotide molecule.

16. A genetically engineered host cell of Claim 13 wherein said cell is suitable for human implantation.

17. A host cell of Claim 15 or 16 wherein said cell is enclosed in a semipermeable membrane suitable for human implantation.

18. A method for the production of a protein product which binds GDNF family receptor-alpha-3 (GFRα-3), said method comprising the steps of:

(a) culturing a genetically engineered host cell comprising a polynucleotide molecule according to claim 5. 6. 7 or 8, under conditions suitable for the expression of said protein product by said host cell; and

(b) optionally, isolating said protein product expressed by said host cell.

19. A method for the production of a protein product which binds GDNF family receptor-alpha-3 (GFRα-3), said method comprising the steps of:

(a) culturing a genetically engineered host cell comprising a polynucleotide molecule encoding a protein product according to claim 1, under conditions suitable for the expression of said protein product by said host cell; and

(b) optionally, isolating said protein product expressed by said host cell.

20. A method of claim 18, wherein said polynucleotide molecule encodes a protein product comprising:

a) amino acid residues 81 through 224 of Figure 3 (SEQ ID NO:__),

b) amino acid residues 109 through 224 of Figure 3 (SEQ ID NO:__),

c) amino acid residues 112 through 224 of Figure 3 (SEQ ID NO:__),

d) amino acid residues 119 through 224 of Figure 3 (SEQ ID NO:__), or

e) amino acid residues 129 through 224 of Figure 3 (SEQ ID NO:__).

21. A method of claim 18, wherein said polynucleotide molecule encodes a protein product comprising:

a) amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),

b) amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),

c) amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),

d) amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),

e) amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),

f) amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),

g) amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),

h) amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), or

i) amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__).

22. An isolated and purified protein product prepared according to the method of claim 18.

23. An isolated and purified protein product prepared according to the method of claim 19.

24. A protein product which binds GDNF family receptor-alpha-3 (GFRα-3), prepared by a method comprising the steps of:

(a) culturing a genetically engineered host cell containing a polynucleotide molecule selected from the group consisting of:

i) a molecule comprising the nucleotides of Figure 2 (SEQ ID NO:__) or its complement,

ii) a molecule encoding a polypeptide comprising amino acid residues 81 through 224 of Figure 3 (SEQ ID NO:__),

iii) a molecule encoding a polypeptide comprising amino acid residues 109 through 224 of Figure 3 (SEQ ID NO:__),

iv) a molecule encoding a polypeptide comprising amino acid residues 112 through 224 of Figure 3 (SEQ ID NO:__),

v) a molecule encoding a polypeptide comprising amino acid residues 119 through 224 of Figure 3 (SEQ ID NO:__),

vi) a molecule encoding a polypeptide comprising amino acid residues 129 through 224 of Figure 3 (SEQ ID NO:__),

vii) a molecule comprising the nucleotides of Figure 6 (SEQ ID NO:__) or its complement,

viii) a molecule encoding a polypeptide comprising amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),

ix) a molecule encoding a polypeptide comprising amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),

x) a molecule encoding a polypeptide comprising amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),

xi) a molecule encoding a polypeptide comprising amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),

xii) a molecule encoding a polypeptide comprising amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),

xiii) a molecule encoding a protein product comprising an amino acid sequence of Figure 3 (SEQ ID NO:__),

xiv) a molecule encoding a protein product comprising an amino acid sequence of Figure 7 (SEQ ID NO:__), and

xv) a molecule encoding a protein product comprising an amino acid sequence of Figure 18 (SEQ ID NO:__)

xvi) a molecule encoding a protein product comprising an amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),

xvii) a molecule encoding a protein product comprising an amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),

xviii) a molecule encoding a protein product comprising an amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), and

xix) a molecule encoding a protein product comprising an amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__)

under conditions suitable for the expression of said protein product by said host cell; and

(b) optionally, isolating said protein product expressed by said host cell.

25. An antibody that binds to a peptide comprising an amino acid sequence of Figure 3 (SEQ ID NO:__), Figure 7 (SEQ ID NO:__) or Figure 18 (SEQ ID NO:__).

26. The antibody of claim 25 wherein said antibody is a monoclonal antibody.

27. The antibody of claim 25 wherein said antibody is a polyclonal antibody.

28. An antibody produced by immunizing an animal with a peptide comprising an amino acid sequence of Figure 3 (SEQ ID NO:__), Figure 7 (SEQ ID NO:__) or Figure 18 (SEQ ID NO:__).

29. A hybridoma that produces a monoclonal antibody that binds to a peptide comprising an amino acid sequence of Figure 3 (SEQ ID NO:__), Figure 7 (SEQ ID NO:__) or Figure 18 (SEQ ID NO:__).

30. A device, comprising:

(a) a membrane suitable for implantation; and

(b) cells encapsulated within said membrane, wherein said cells secrete a protein product of claim 1;

said membrane being permeable to said protein product and impermeable to materials detrimental to said cells.

31. A device, comprising:

(a) a membrane suitable for implantation; and

(b) cells encapsulated within said membrane, wherein said cells contain a polynucleotide molecule selected from the group consisting of:

i) a molecule comprising the nucleotides of Figure 2 (SEQ ID NO:__),

ii) a molecule comprising the nucleotides of Figure 6 (SEQ ID NO:__),

iii) a molecule encoding a protein product comprising an amino acid residues 81 through 228 of Figure 7 (SEQ ID NO:__),

iv) a molecule encoding a protein product comprising an amino acid residues 89 through 228 of Figure 7 (SEQ ID NO:__),

v) a molecule encoding a protein product comprising an amino acid residues 113 through 228 of Figure 7 (SEQ ID NO:__),

vi) a molecule encoding a protein product comprising an amino acid residues 116 through 228 of Figure 7 (SEQ ID NO:__),

vii) a molecule encoding a protein product comprising an amino acid residues 133 through 228 of Figure 7 (SEQ ID NO:__),

viii) a molecule encoding a protein product comprising an amino acid residues PPP through CLG of Figure 18 (SEQ ID NO:__),

ix) a molecule encoding a protein product comprising an amino acid residues AAR through CLG of Figure 18 (SEQ ID NO:__),

x) a molecule encoding a protein product comprising an amino acid residues AGXaa through CLG of Figure 18 (SEQ ID NO:__), and

xi) a molecule encoding a protein product comprising an amino acid residues LRS through CLG of Figure 18 (SEQ ID NO:__),

wherein said cells express and secrete said protein product,

and wherein said membrane is permeable to said protein product and impermeable to materials detrimental to said cells.

32. The use of the isolated and purified protein product of claim 1 for the manufacture of a pharmaceutical composition.

33. A pharmaceutical composition comprising a protein product of claim 1 in combination with a pharmaceutically acceptable carrier.

ABSTRACT

The present invention relates to novel molecules referred to as GDNF-related neurotrophic factor 4 (GRNF4). The present invention involves the cloning, expression, characterization and use of GRNF4. Polynucleotide molecules and amino acid sequences are described for GRNF4. GRNF4 is 35% identical to glial cell line-derived neurotrophic factor and 46% identical to neurturin. GRNF4 binds to GFRα-3 which is a receptor of the GFRα family exclusively expressed in the peripheral sensory and sympathetic nervous systems.

Docket No.: A-574P

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am a joint inventor of the invention entitled

GRNF4 A NEUROTROPHIC FACTOR

which is described and claimed in the specification which:

☒ is attached hereto.

☐ was filed on _____
as Application Serial No. _____
and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

Power of Attorney: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Ron K. Levy, Registration No.: 31,539, Steven M. Odre, Registration No.: 29,094, and Daniel R. Curry, Registration No.: 32,727, said attorneys to have in addition full power of revocation, including the power to revoke any power herein granted.

Please send all future correspondence to:

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One Amgen Center Drive
Thousand Oaks, California 91320-1799

Direct Telephone Calls To:

Daniel R. Curry
Attorney for Applicants
Registration No.: 32,727
Phone: (805) 447-8102

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Printed Name

Signature

DECLARATION AND POWER OF ATTORNEY (cont'd)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of First Inventor: W. Scott Simonet

Inventor's Signature: W. Scott Simonet Date: 9-Dec. 98

Residence and Post Office Address: 2293 Watertown Court
Thousand Oaks, California 91360 USA
(Address, City, State, Zip Code, Country)

Citizenship: U.S.

Full Name of Second Joint Inventor, if Any: Franklin J. Asuncion

Inventor's Signature: Date: 9 Dec 98

Residence and Post Office Address: 2279 McDonald Street
Simi Valley, California 93065 USA
(Address, City, State, Zip Code, Country)

Citizenship: U.S.

Full Name of Third Joint Inventor, if Any: Hosung Min

Inventor's Signature: Date: 12-9-98

Residence and Post Office Address: 5811 Woodman Avenue, #1
Sherman Oaks, California 91401 USA
(Address, City, State, Zip Code, Country)

Citizenship: U.S.

DECLARATION AND POWER OF ATTORNEY (cont'd)

Full Name of Fourth Joint Inventor, if Any: Shuqian Jing

Inventor's Signature: _____ Date: 12-9-98

Residence and Post Office Address: 3254 Bordero Lane
Thousand Oaks, California 91362 USA
(Address, City, State, Zip Code, Country)

Citizenship: China

Figure 1a

```
  1 CGCGTGGGCG CGTGCAGGAA CCCGGAGCAG CCGCGCACGG ACCACAGATG
 51 CGCGCGGCTG CCGCCTGCGC TCGCAGCTGG TGCCGGTGAG TGCGCTCGGC
101 CTAGGCCACA GCTCCGACGA GCTGATACGT TTCCGCTTCT GCAGCGGCTC
151 GTGCCGTCGA GCACGCTCCC AGCACGATCT CAGTCTGGCC AGCCTACTGG
201 GCGCTGGGGC CCTACGGTCG CCTCCCGGGT CCCGGNCGAT CAGCCAGCCC
251 TGCTGCCGGC CCACTCGCTA TGAGGCCGTC TCCTTCATGG ACGTGAACAG
301 CACCTGNAGG ACCGTGGACC ACCTCTCCGC CACTGCCTGC GGCTGTCTGG
351 GCTGAGGATG ATCTATCTCC AAGCCTTT
```

Figure 1b

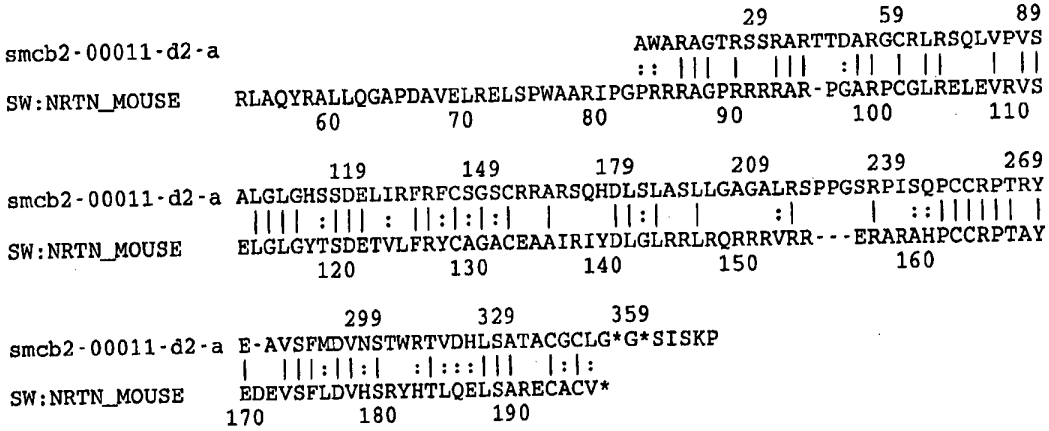

Figure 1c

```
   1 CGGACGCGTG GGCGGACGCG TGGGCGCGTG CAGGAACCCG GAGCAGCCGC
  51 GCACGGACCA CAGATGCGCG CGGCTGCCGC CTGCGCTCGC AGCTGGTGCC
 101 GGTGAGTGCG CTCGGCCTAG GCCACAGCTC CGACGAGCTG ATACGTTTCC
 151 GCTTCTGCAG CGGCTCGTGC CGCCGAGCAC GCTCCCAGCA CGATCTCAGT
 201 CTGGCCAGCC TACTGGGCGC TGGGGCCCTA CGGTCGCCTC CCGGGTCCCG
 251 GCCGATCAGC CAGCCCTGCT GCCGGCCCAC TCGCTATGAG GCCGTCTCCT
 301 TCATGGACGT GAACAGCACC TGGAGGACCG TGGACCACCT CTCCGCCACT
 351 GCCTGCGGCT GTCTGGGCTG AGGATGATCT ATCTCCAAGC CTTTGCACAC
 401 TAGACCCATG TGTTGCCCTA CCTGGAACAG CTCCACCGGG CCTCACTAAC
 451 CAGGAGCCTC AACTCAGCAG GATATGGAGG CTGCAGAGCT CAGGCCCCAG
 501 GCCGGTGAGT GACAGACGTC GTCGGCATGA CAGACAGAGT GAAAGATGTC
 551 GGAACCACTG ACCAACAGTC CCAAGTTGTT CATGGATCAC AGCTCTACAG
 601 ACAGGAGAAA CCTCAGCTAA AGAGAACTCC TCTGGGAGAA TCCAGAAATG
 651 GCCCTCTGTC CTGGGGAATG AATTTTGAAG AGATATATAT ACATATATAC
 701 ATTGTAGTCG CGTTGCTGGA CCAGCCTGTG CTGAAACCAG TCCCGTGTTC
 751 ACTTGTGGAA GCCGAAGCCC TATTTATTAT TTCTAAATTA TTTATTTACT
 801 TTGCTGGTTT GTCAGATCCT TTCCTGGACA TGGGGGATGG TAGAAGAAGC
 851 TAGATGAAGA TGTGCCCCAC CCCACCCCCC CATCCACATT TTACACTTGA
 901 CTCAGTAGTG CTACCTGGAT CGCCTACTTC TTGCCCCGCA GGTGTCTCTG
 951 AGATGGATGG GAGGCACACA TAGGTGACAA AGATGCACAA TCCACAGTAC
1001 TTGGGGCCTG GGGTACCTAT GGGAAATAAA CAATATAGTT TTCTATGGAA
1051 AAAA
```

Figure 2

1 CCAAGCTTGG TACCGAGCTC GGATCCACTA GTAACGGCCG CCAGTGTGCT
51 GGAATTCGCC CTTACTCACT ATAGGGCTCG AGCGGCCGCC CGGGCAGGTA
101 TAAAAAAAAA AAGCGGCCTA GAATTCAGCG GCCGCTGAAT TCTAGGCTGC
151 CGCAGGAAGA GGGTGGGGAA ACGGGTCCAC GAAGGCTTCT GATGGGAGCT
201 TCTGGAGCCG AAAGCTATGG AACTGGGACT TGCAGAGCCT ACTGCATTGT
251 CCCACTGCCT CCGGCCTAGG TGGCAGTCAG CCTGGTGGCC AACCCTAGCT
301 GTTCTAGCCC TGCTGAGCTG CGTCACAGAA GCTTCCCTGG ACCCAATGTC
351 CCGCAGCCCC GCCGCTCGCG ACGGTCCCTC ACCGGTCTTG GCGCCCCCCA
401 CGGACCACCT GCCTGGGGGA CACACTGCGC ATTTGTGCAG CGAAAGAACC
451 CTGCGACCCC CGCCTCAGTC TCCTCAGCCC GCACCCCCGC CGCCTGGTCC
501 CGCGCTCCAG TCTCCTCCCG CTGCGCTCCG CGGGGCACGC GCGGCGCGTG
551 CAGGAACCCG GAGCAGCCGC GCACGGACCA CAGATGCGCG CGGCTGCCGC
601 CTGCGCTCGC AGCTGGTGCC GGTGAGCGCG CTCGGCCTAG GCCACAGCTC
651 CGACGAGCTG ATACGTTTCC GCTTCTGCAG CGGCTCGTGC CGCCGAGCAC
701 GCTCCCAGCA CGATCTCAGT CTGGCCAGCC TACTGGGCGC TGGGGCCCTA
751 CGGTCGCCTC CCGGGTCCCG GCCGATCAGC CAGCCCTGCT GCCGGCCCAC
801 TCGCTATGAG GCCGTCTCCT TCATGGACGT GAACAGCACC TGGAGGACCG
851 TGGACCACCT CTCCGCCACT GCCTGCGGCT GTCTGGGCTG AGGATGATCT
901 ATCTCCAAGC CTTTGCACAC TAGACCCATG TGTTGCCCTA CCTGGAACAG
951 CTCCAAGGGC GAATTCTGCA GATATCCATC ACACTGGCGG CCGCTCGAGC
1001 ATGCATCTAG AGG

Figure 3

```
  1  MELGLAEPTA LSHCLRPRWQ SAWWPTLAVL ALLSCVTEAS LDPMSRSPAA
 51  RDGPSPVLAP PTDHLPGGHT AHLCSERTLR PPPQSPQPAP PPPGPALQSP
101  PAALRGARAA RAGTRSSRAR TTDARGCRLR SQLVPVSALG LGHSSDELIR
151  FRFCSGSCRR ARSQHDLSLA SLLGAGALRS PPGSRPISQP CCRPTRYEAV
201  SFMDVNSTWR TVDHLSATAC GCLG*
```

Figure 4

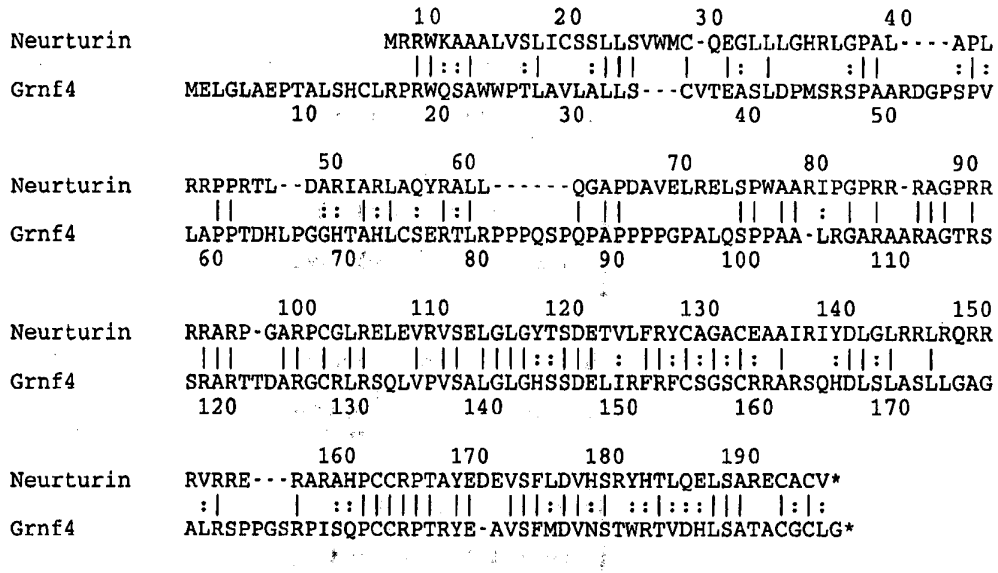

Figure 5

ClustalW Formatted Alignments

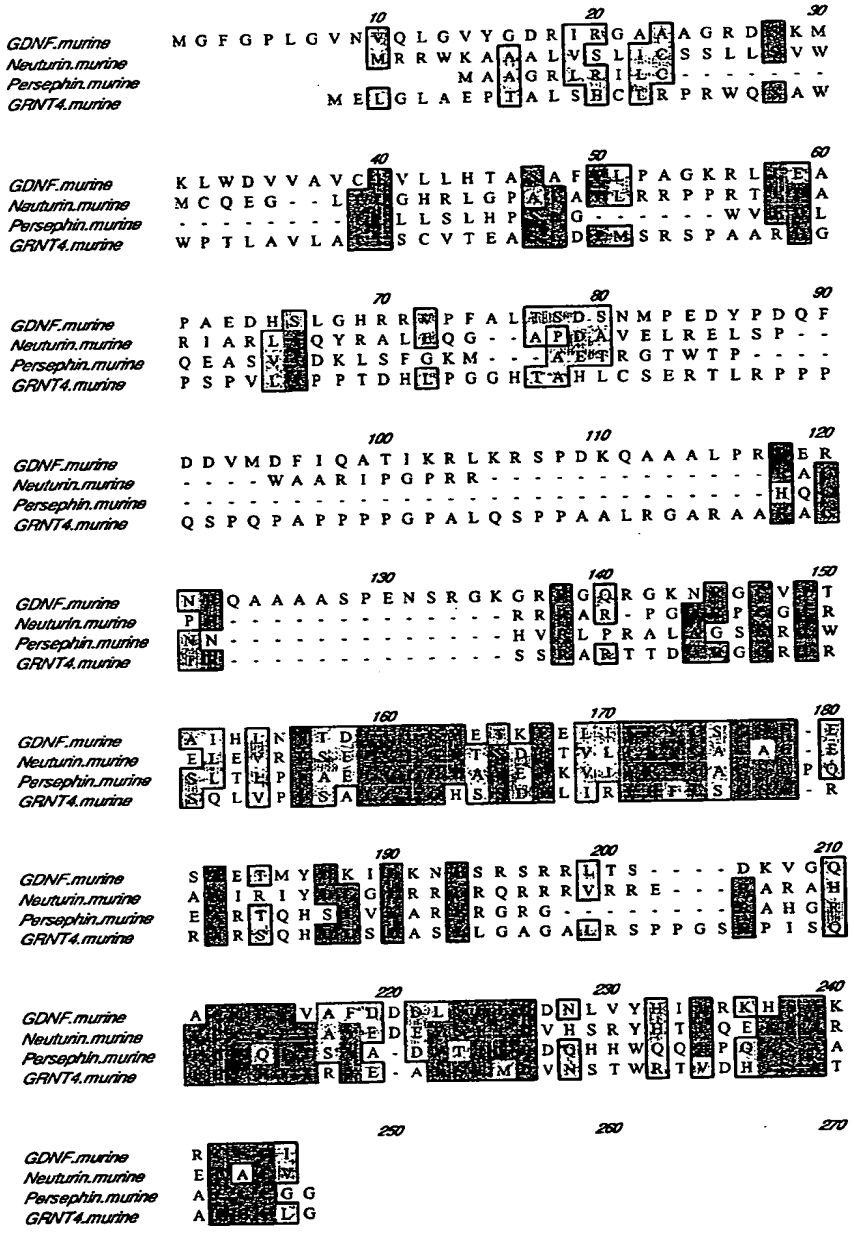

Figure 6

```
  1  CAACAATGGC TGATGGGCGC TCCTGGTGTT GATAGAGATG GAACTTGGAC
 51  TTGGAGGCCT CTCCACGCTG TCCCACTGCC CCTGGCCTAG GCGGCAGGCT
101  CCACTTGGTC TCTCCGCGCA GCCTGCCCTG TGGCCCACCC TGGCCGCTCT
151  GGCTCTGCTG AGCAGCGTCG CAGAGGCCTC CCTGGGCTCC GCGCCCCGCA
201  GCCCTGCCCC CCGCGAAGGC CCCCCGCCTG TCCTGGCGTC CCCCGCCGGC
251  CACCTGCCGG GGGGACGCAC GGCCCGCTGG TGCAGTGGAA GAGCCCGGCG
301  GCCGCCGCCG CAGCCTTCTC GGCCCGCGCC CCCGCCGCCT GCACCCCCAT
351  CTGCTCTTCC CCGCGGGGGC CGCGCGGCGC GGGCTGGGGG CCCGGGCAGC
401  CGCGCTCGGG CAGCGGGGGC GCGGGGCTGC CGCCTGCGCT CGCAGCTGGT
451  GCCGGTGCGC GCGCTCGGCC TGGGCCACCG CTCCGACGAG CTGGTGCGTT
501  TCCGCTTCTG CAGCGGCTCC TGCCGCCGCG CGCGCTCTCC ACACGACCTC
551  AGCCTGGCCA GCCTACTGGG CGCCGGGGCC CTGCGACCGC CCCCGGGCTC
601  CCGGCCCGTC AGCCAGCCCT GCTGCCGACC CACGCGCTAC GAAGCAGTCT
651  CCTTCATGGA CGTCAACAGC ACCTGGAGAA CCGTGGACCG CCTCTCCGCC
701  ACCGCCTGCG GCTGCCTGGG CTGAGGGCTC GCTCCA
```

Figure 7

```
1    MELGLGGLST LSHCPWPRRQ APLGLSAQPA LWPTLAALAL LSSVAEASLG
51   SAPRSPAPRE GPPPVLASPA GHLPGGRTAR WCSGRARRPP PQPSRPAPPP
101  PAPPSALPRG GRAARAGGPG SRARAAGARG CRLRSQLVPV RALGLGHRSD
151  ELVRFRFCSG SCRRARSPHD LSLASLLGAG ALRPPPGSRP VSQPCCRPTR
201  YEAVSFMDVN STWRTVDRLS ATACGCLG*
```

Figure 8

```
mouse     1 MELGLAEPTALSHCLRPRWQS........AWWPTLAVLALLSCVTEASLD 42
            |||||    .||||  || |.        | ||||| ||||| | ||||
human     1 MELGLGGLSTLSHCPWPRRQAPLGLSAQPALWPTLAALALLSSVAEASLG 50
                     .         .         .         .         .
mouse    43 PMSRSPAARDGPSPVLAPPTDHLPGGHTAHLCSERTLRPPPQSPQPAPPP 92
               |||| |:|| |||| |  ||||| ||  || |  |||||  .|||||
human    51 SAPRSPAPREGPPPVLASPAGHLPGGRTARWCSGRARRPPPQPSRPAPPP 100
                     .         .         .         .         .
mouse    93 PGPALQSPPAALRGARAARAGTRSSRARTTDARGCRLRSQLVPVSALGLG 142
            | |    | |  || ||||||   ||||   ||||||||||||| |||||
human   101 PAP....PSALPRGGRAARAGGPGSRARAAGARGCRLRSQLVPVRALGLG 146
                     .         .         .         .         .
mouse   143 HSSDELIRFRFCSGSCRRARSQHDLSLASLLGAGALRSPPGSRPISQPCC 192
            | ||||:|||||||||||||| ||||||||||||||| ||||||:|||||
human   147 HRSDELVRFRFCSGSCRRARSPHDLSLASLLGAGALRPPPGSRPVSQPCC 196
                     .         .         .
mouse   193 RPTRYEAVSFMDVNSTWRTVDHLSATACGCLG* 225
            ||||||||||||||||||||| ||||||||||
human   197 RPTRYEAVSFMDVNSTWRTVDRLSATACGCLG* 229
```

Figure 9

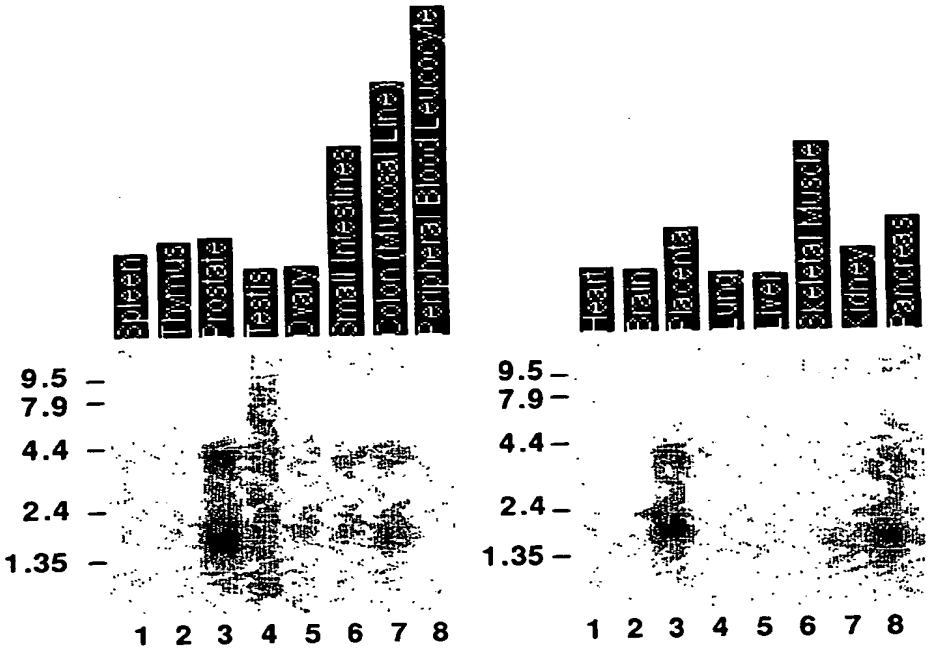

Figure 10

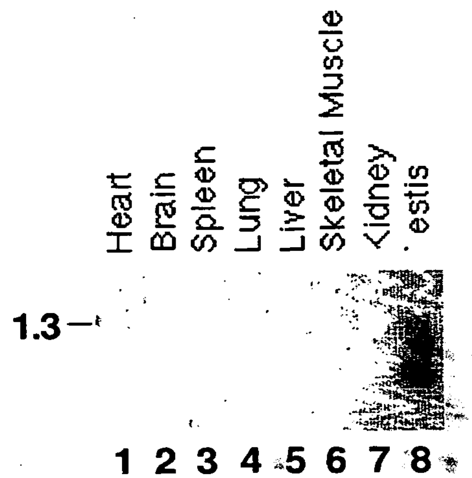
Heart
Brain
Spleen
Lung
Liver
Skeletal Muscle
Kidney
estis
1.3
1 2 3 4 5 6 7 8

Figure 11

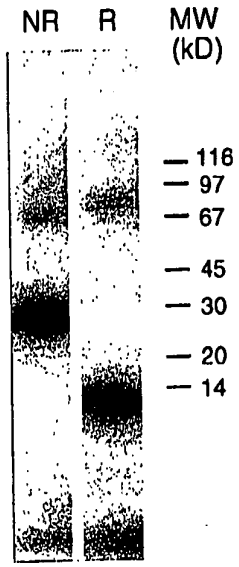

Figure 12

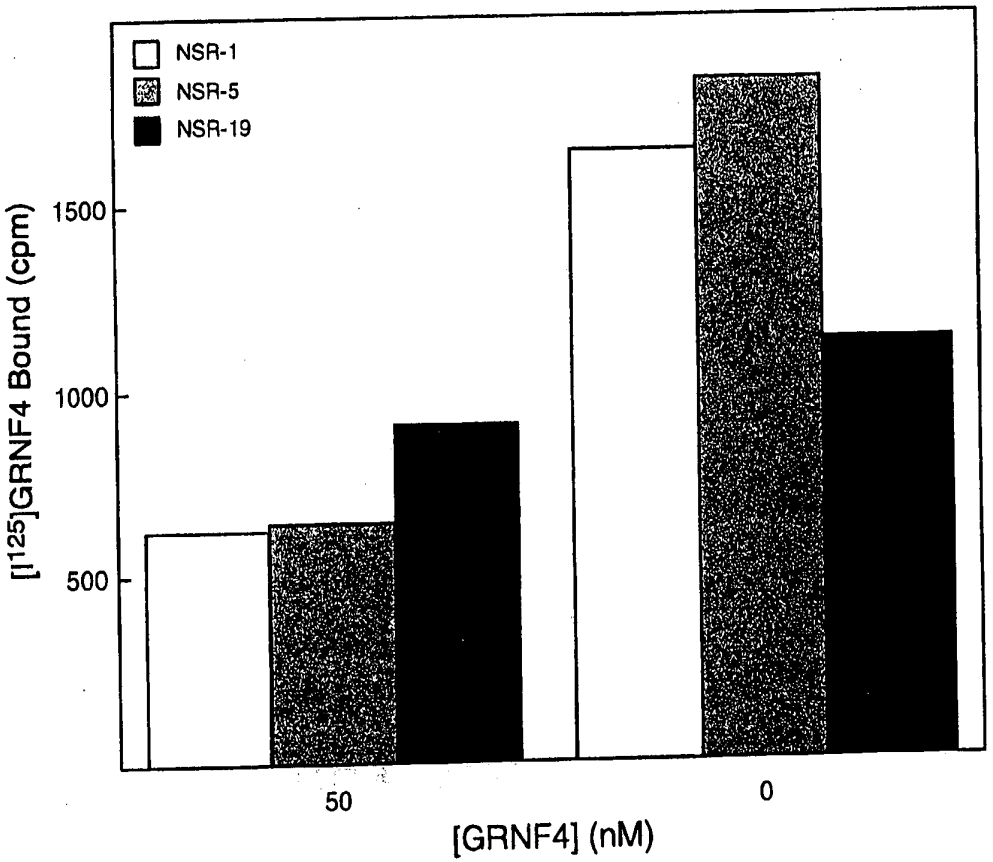

Figure 13

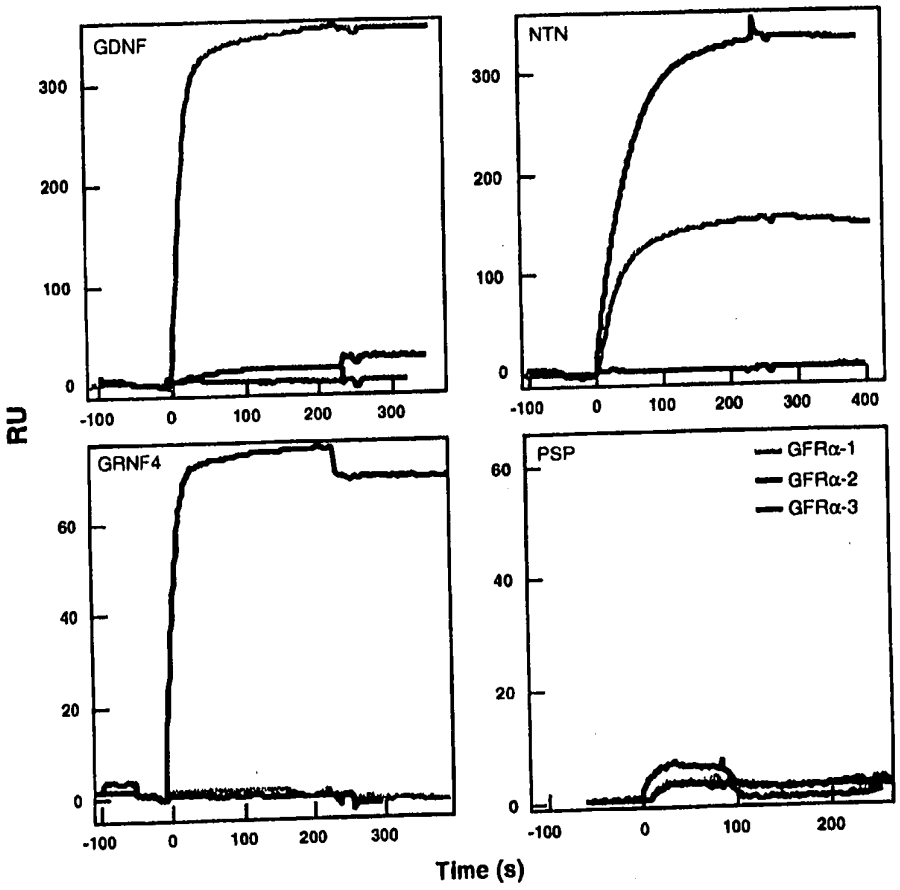

Figure 14

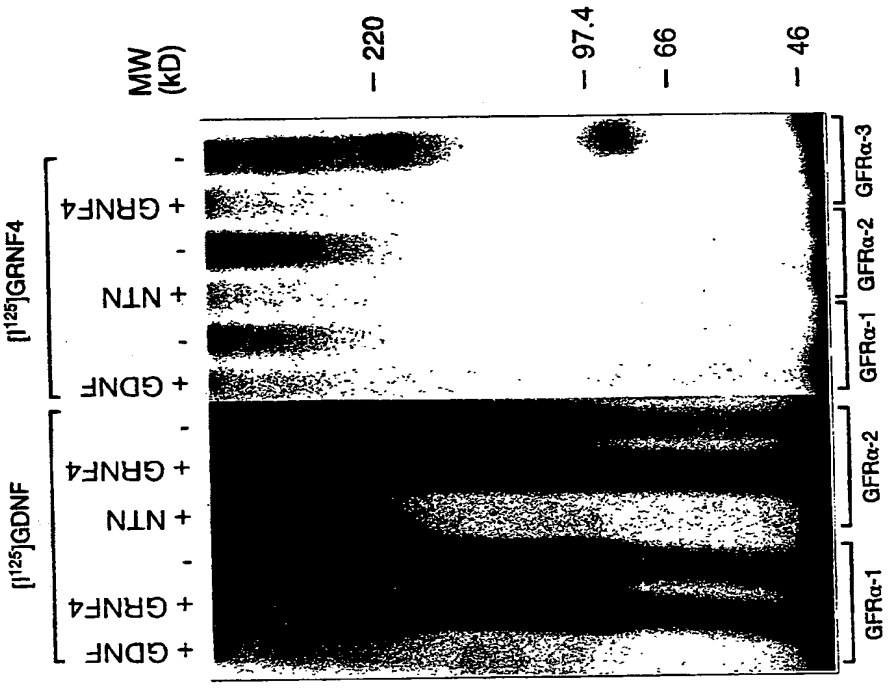
MW (kD)
220
97.4
66
46
[I125]GDNF
+ GDNF
+ GRNF4
-
+ NTN
-
+ GRNF4
-
[I125]GRNF4
+ GDNF
+ NTN
-
+ GRNF4
-
GFRα-1
GFRα-2
GFRα-1
GFRα-2
GFRα-3

Figure 15

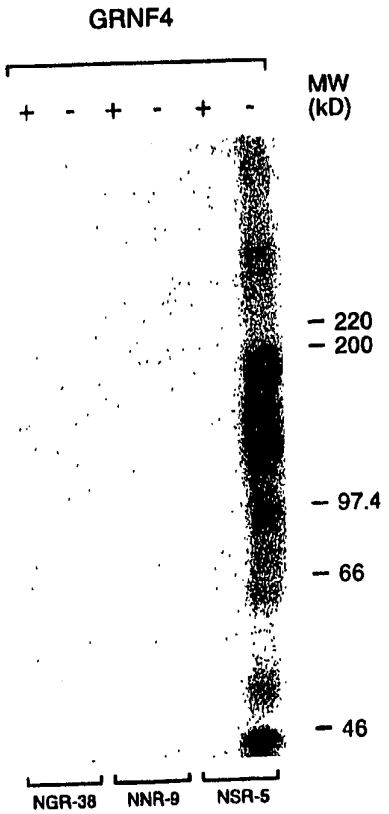

Figure 16

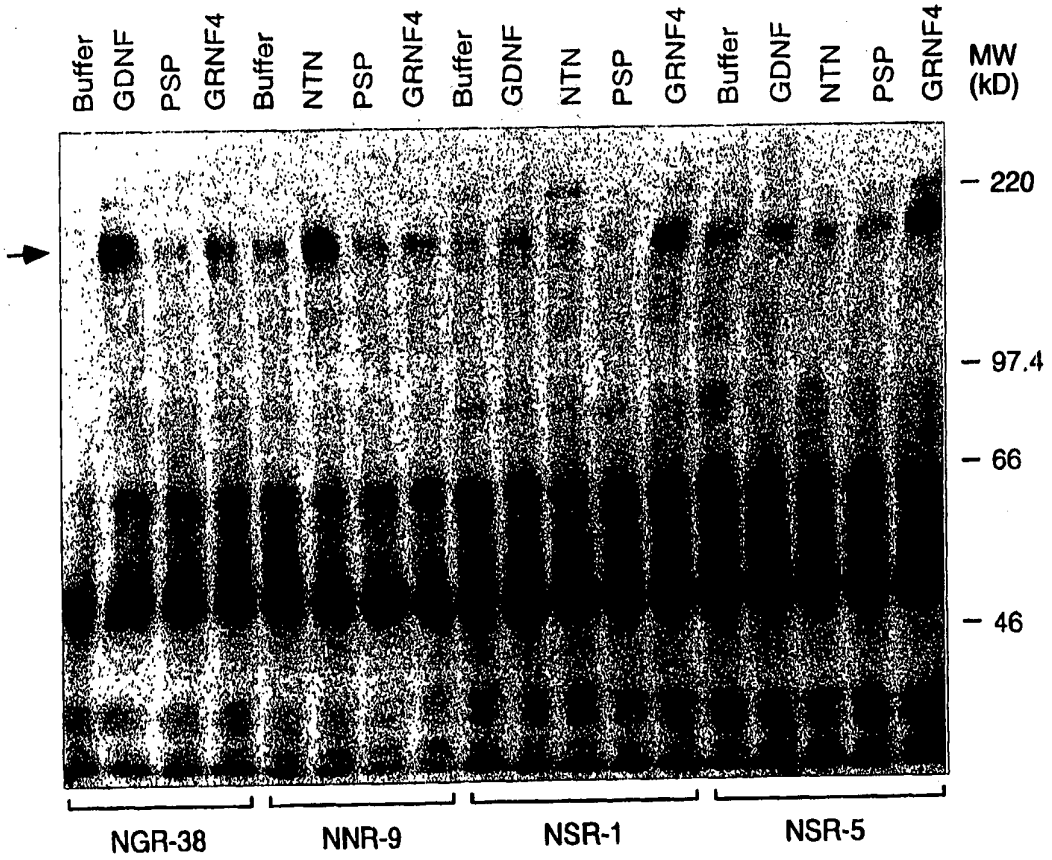

Figure 17

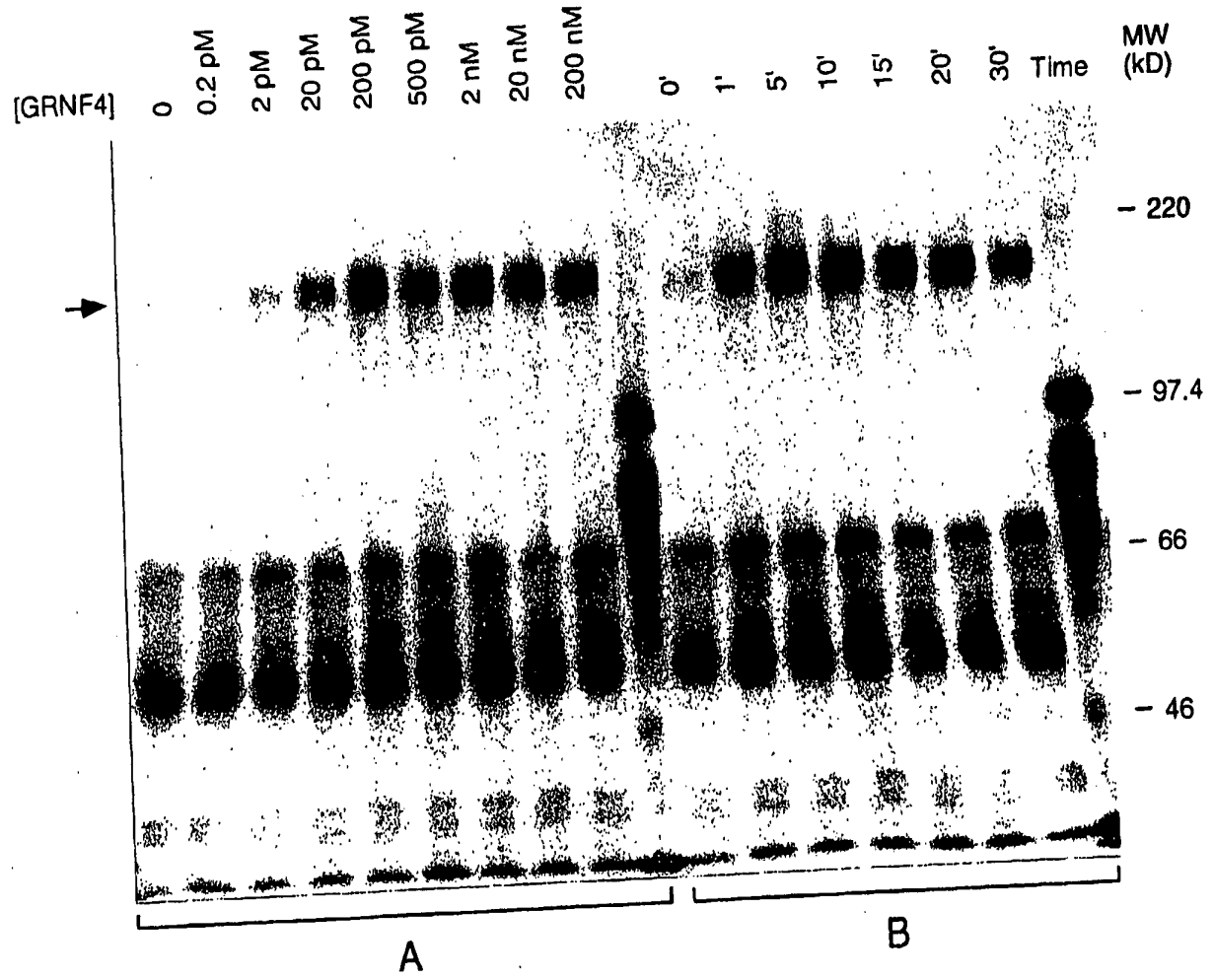

Figure 18

M E L G L [Xaa]$_5$ L S H C [Xaa]$_2$ P R **[Xaa]$_{0-8}$** [Xaa] Q [Xaa] A [Xaa] W P T L A [Xaa] L A L L S [Xaa] V [Xaa] E A S L [Xaa]$_4$ R S P A [Xaa] R [Xaa] G P [Xaa] P V L A [Xaa] P [Xaa]$_2$ H L P G G [Xaa] T A [Xaa]$_2$ C S [Xaa] R [Xaa]$_2$ R P P P Q [Xaa]$_3$ P A P P P P [Xaa] P [Xaa]$_4$ P **[Xaa]$_{0-4}$** R G [Xaa] R A A R A G [Xaa]$_3$ S R A R [Xaa]$_3$ A R G C R L R S Q L V P V [Xaa] A L G L G H [Xaa] S D E L [Xaa] R F R F C S G S C R R A R S [Xaa] H D L S L A S L L G A G A L R [Xaa] P P G S R P [Xaa] S Q P C C R P T R Y E A V S F M D V N S T W R T V D [Xaa] L S A T A C G C L G